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(54) Title: RECOMBINANT VASCULAR ENDOTHELIAL CELL GROWTH FACTOR D (VEGF-D)

(57) Abstract

VEGF-D, a new member of the PDGF family of growth factors, which among other things stimulates endothelial cell proliferation and angiogenesis and increases vascular permeability, is described, as well as nucleotide sequences encoding it, methods for producing it, antibodies and other antagonists to it, transfected or transformed host cells for expressing it, pharmaceutical compositions containing it, and uses thereof in medical and diagnostic applications.

RECOMBINANT VASCULAR ENDOTHELIAL CELL GROWTH FACTOR D (VEGF-D)

This invention relates to growth factors for endothelial cells, and in particular to a novel vascular endothelial growth factor, DNA encoding the factor, and to pharmaceutical and diagnostic compositions and methods utilising or derived from the factor.

BACKGROUND OF THE INVENTION

Angiogenesis is a fundamental process required for normal growth and development of tissues, and involves the proliferation of new capillaries from pre-existing blood vessels. Angiogenesis is not only involved in embryonic development and normal tissue growth, repair, and regeneration, but is also involved in the female reproductive cycle, establishment and maintenance of pregnancy, and in repair of wounds and fractures. In addition to angiogenesis which takes place in the normal individual, angiogenic events are involved in a number of pathological processes, notably tumour growth and metastasis, and other conditions in which blood vessel proliferation, especially of the microvascular system, is increased, such as diabetic retinopathy, psoriasis and arthropathies. Inhibition of angiogenesis is useful in preventing or alleviating these pathological processes.

On the other hand, promotion of angiogenesis is desirable in situations where vascularization is to be established or extended, for example after tissue or organ transplantation, or to stimulate establishment of collateral

circulation in tissue infarction or arterial stenosis, such as in coronary heart disease and thromboangitis obliterans.

Because of the crucial role of angiogenesis in so many physiological and pathological processes, factors involved in the control of angiogenesis have been intensively investigated. A number of growth factors have been shown to be involved in the regulation of angiogenesis; these include fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), transforming growth factor α (TGFα), and hepatocyte growth factor (HGF). See for example Folkman et al, "Angiogenesis", J. Biol. Chem., 1992 267 10931-10934 for a review.

It has been suggested that a particular family of endothelial cell-specific growth factors and their corresponding receptors is primarily responsible for stimulation of endothelial cell growth and differentiation, and for certain functions of the differentiated cells. These factors are members of the PDGF family, and appear to act via endothelial receptor tyrosine kinases (RTKs). Hitherto four vascular endothelial growth factor subtypes have been identified. Vascular endothelial growth factor (VEGF), now known as VEGF-A, has been isolated from several sources. VEGF-A shows highly specific mitogenic activity against endothelial cells, and can stimulate the whole sequence of events leading to angiogenesis. In addition, it has strong chemoattractant activity towards monocytes, can induce plasminogen activator and plasminogen activator inhibitor in endothelial cells, and can also influence microvascular permeability. Because of the latter activity, it is also sometimes referred to as vascular permeability factor (VPF). The isolation and properties of VEGF have been reviewed; see Ferrara et al, "The Vascular Endothelial Growth Factor Family of Polypeptides", J. Cellular Biochem., 1991 47 211-218 and Connolly, "Vascular Permeability Factor: A Unique Regulator of Blood Vessel Function", J. Cellular Biochem., 1991 47 219-223.

More recently, three further members of the VEGF family have been identified. These are designated VEGF-B, described in International Patent Application No. PCT/US96/02957 (WO 96/26736) by Ludwig Institute for Cancer Research and The University of Helsinki, VEGF-C, described in Joukov et al, The EMBO Journal, 1996 15 290-298, and VEGF2, described in International Patent Application No. PCT/US94/05291 (WO 95/24473) by Human Genome Sciences, Inc. VEGF-B has closely similar angiogenic and other properties to those of VEGF, but is distributed and expressed in tissues differently from VEGF. In particular, VEGF-B is very strongly expressed in heart, and only weakly in lung, whereas the reverse is the case for VEGF. This suggests that VEGF and VEGF-B, despite the fact that they are co-expressed in many tissues, may have functional differences.

VEGF-B was isolated using a yeast co-hybrid interaction trap screening technique, screening for cellular proteins which might interact with cellular retinoic acid-binding protein type I (CRABP-I). Its isolation and characteristics are described in detail in PCT/US96/02597 and in Olofsson et al, Proc. Natl. Acad. Sci., 1996 93 2576-2581.

VEGF-C was isolated from conditioned media of PC-3 prostate adenocarcinoma cell line (CRL1435) by screening for ability of the medium to produce tyrosine phosphorylation of the endothelial cell-specific receptor tyrosine kinase Flt4, using cells transfected to express Flt4. VEGF-C was purified using affinity chromatography with recombinant Flt4, and was cloned from a PC-3 cDNA library. Its isolation and characteristics are described in detail in Joukov et al, The EMBO Journal, 1996 15 290-298.

VEGF2 was isolated from a highly tumorgenic, oestrogen-independent human breast cancer cell line. While this molecule is stated to have about 22% homology to PDGF and 30% homology to VEGF, the method of isolation of the gene encoding VEGF2 is unclear, and no characterization of the biological activity is disclosed.

Vascular endothelial growth factors appear to act by binding to receptor tyrosine kinases of the PDGF-receptor family. Five endothelial cell-specific receptor tyrosine kinases have been identified, namely Flt-1 (VEGFR-1),
5   KDR/Flk-1 (VEGFR-2), Flt4 (VEGFR-3), Tie and Tek/Tie-2. All of these have the intrinsic tyrosine kinase activity which is necessary for signal transduction. The essential, specific role in vasculogenesis and angiogenesis of Flt-1, Flk-1, Tie and Tek/Tie-2 has been demonstrated by targeted
10   mutations inactivating these receptors in mouse embryos. VEGFR-1 and VEGFR-2 bind VEGF with high affinity, and VEGFR-1 also binds VEGF-B and placenta growth factor (PlGF). VEGF-C has been shown to be the ligand for Flt4 (VEGFR-3), and also activates VEGFR-2 (Joukov et al, 1996). A ligand
15   for Tek/Tie-2 has been described (International Patent Application No. PCT/US95/12935 (WO 96/11269) by Regeneron Pharmaceuticals, Inc.); however, the ligand for Tie has not yet been identified.

The receptor Flt-4 is expressed in venous and lymphatic
20   endothelia in the fetus, and predominantly in lymphatic endothelia in the adult (Kaipainen et al, Cancer Res, 1994 54 6571-6577; Proc Natl. Acad. Sci. USA, 1995 92 3566-3570). It has been suggested that VEGF-C may have a primary function in lymphatic endothelium, and a secondary function
25   in angiogenesis and permeability regulation which is shared with VEGF (Joukov et al, 1996).

We have now isolated human cDNA encoding a novel protein of the vascular endothelial growth factor family. The novel protein, designated VEGF-D, has structural
30   similarities to other members of this family.

SUMMARY OF THE INVENTION

The invention generally provides an isolated novel growth factor which has the ability to stimulate and/or
35   enhance proliferation or differentiation of endothelial cells, isolated DNA sequences encoding the novel growth

factor, and compositions useful for diagnostic and/or therapeutic applications.

According to one aspect, the invention provides an isolated and purified nucleic acid molecule which encodes a
5 novel polypeptide, designated VEGF-D, which is structurally homologous to VEGF, VEGF-B and VEGF-C. In a preferred embodiment, the nucleic acid molecule is a cDNA which comprises the sequence set out in SEQ ID NO. 1, SEQ ID NO. 4, SEQ ID NO. 6 or SEQ ID NO. 7. This aspect of
10 the invention also encompasses DNA molecules of sequence such that they hybridise under stringent conditions with DNA of SEQ ID NO. 1, SEQ ID NO. 4, SEQ ID NO. 6 or SEQ ID NO. 7. Preferably the DNA molecule able to hybridise under stringent conditions encodes the portion of VEGF-D from
15 amino acid residue 93 to amino acid residue 201, optionally operatively linked to a DNA sequence encoding FLAG™ peptide.

Preferably the cDNA comprises the sequence set out in SEQ ID NO. 4, SEQ ID NO. 6 or SEQ ID NO. 7, more preferably that of SEQ ID NO. 4.
20 According to a second aspect, the invention provides a polypeptide possessing the characteristic amino acid sequence:

Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys
25 (SEQ ID NO. 2),

said polypeptide having the ability to stimulate proliferation of endothelial cells, and said polypeptide comprising a sequence of amino acids substantially
30 corresponding to the amino acid sequence set out in SEQ ID NO. 3, or a fragment or analogue thereof which has the ability to stimulate one or more of endothelial cell proliferation, differentiation, migration or survival.

These abilities are referred to herein as "biological
35 activities of VEGF-D" and can readily be tested by methods known in the art. Preferably the polypeptide has the

ability to stimulate endothelial cell proliferation or differentiation, including, but not limited to, proliferation or differentiation of vascular endothelial cells and/or lymphatic endothelial cells.

More preferably the polypeptide has the sequence set out in SEQ ID NO. 5, SEQ ID NO. 8 or SEQ ID NO. 9, and most preferably has the sequence set out in SEQ ID NO. 5.

A preferred fragment of the polypeptide invention is the portion of VEGF-D from amino acid residue 93 to amino acid residue 201, optionally linked to FLAG™ peptide. Where the fragment is linked to FLAG™, the fragment is VEGFDΔNΔC, as hereindefined.

Thus polypeptides comprising conservative substitutions, insertions, or deletions, but which still retain the biological activity of VEGF-D, are clearly to be understood to be within the scope of the invention. The person skilled in the art will be well aware of methods which can readily be used to generate such polypeptides, for example the use of site-directed mutagenesis, or specific enzymic cleavage and ligation. The skilled person will also be aware that peptidomimetic compounds or compounds in which one or more amino acid residues are replaced by a non-naturally occurring amino acid or an amino acid analogue may retain the required aspects of the biological activity of VEGF-D. Such compounds can readily be made and tested by methods known in the art, and are also within the scope of the invention.

In addition, variant forms of the VEGF-D polypeptide which result from alternative splicing, as are known to occur with VEGF, and naturally-occurring allelic variants of the nucleic acid sequence encoding VEGF-D are encompassed within the scope of the invention. Allelic variants are well known in the art, and represent alternative forms or a nucleic acid sequence which comprise substitution, deletion or addition of one or more nucleotides, but which do not

result in any substantial functional alteration of the encoded polypeptide.

As used herein, the term "VEGF-D" collectively refers to the polypeptides of SEQ ID NO. 3, SEQ ID NO. 5, SEQ ID NO. 8 and SEQ ID NO. 9 and fragments or analogues thereof which have the biological activity of VEGF-D as herein defined.

Such variant forms of VEGF-D can be prepared by targeting non-essential regions of the VEGF-D polypeptide for modification. These non-essential regions are expected to fall outside the strongly-conserved regions indicated in the figures herein, especially Figure 2 and Figure 10. In particular, the growth factors of the PDGF family, including VEGF, are dimeric, and VEGF-B, VEGF-C, PlGF, PDGF-A and PDGF-B show complete conservation of 8 cysteine residues in the N-terminal domains, ie. the PDGF-like domains (Olofsson et al, 1996; Joukov et al, 1996). These cysteines are thought to be involved in intra- and inter-molecular disulphide bonding. In addition there are further strongly, but not completely, conserved cysteine residues in the C-terminal domains. Loops 1, 2 and 3 of each subunit, which are formed by intra-molecular disulphide bonding, are involved in binding to the receptors for the PDGF/VEGF family of growth factors (Andersson et al: Growth Factors, 1995 12 159-164) As shown herein, the cysteines conserved in previously known members of the VEGF family are also conserved in VEGF-D.

The person skilled in the art thus is well aware that these cysteine residues should be preserved in any proposed variant form, and that the active sites present in loops 1, 2 and 3 also should be preserved. However, other regions of the molecule can be expected to be of lesser importance for biological function, and therefore offer suitable targets for modification. Modified polypeptides can readily be tested for their ability to show the biological activity of

VEGF-D by routine activity assay procedures such as cell proliferation tests.

It is contemplated that some modified VEGF-D polypeptides will have the ability to bind to endothelial cells, ie. to VEGF-D receptors, but will be unable to stimulate endothelial cell proliferation, differentiation, migration or survival. These modified polypeptides are expected to be able to act as competitive or non-competitive inhibitors of VEGF-D, and to be useful in situations where prevention or reduction of VEGF-D action is desirable. Thus such receptor-binding but non-mitogenic, non-differentiation inducing, non-migration inducing or non-survival promoting variants of VEGF-D are also within the scope of the invention, and are referred to herein as "receptor-binding but otherwise inactive variants".

According to a third aspect, the invention provides a purified and isolated nucleic acid encoding a polypeptide or polypeptide fragment of the invention. The nucleic acid may be DNA, genomic DNA, cDNA or RNA, and may be single-stranded or double stranded. The nucleic acid may be isolated from a·cell or tissue source, or of recombinant or synthetic origin. Because of the degeneracy of the genetic code, the person skilled in the art will appreciate that many such coding sequences are possible, where each sequence encodes the amino acid sequence shown in SEQ ID NO. 3, SEQ ID NO. 5, SEQ ID NO. 8 or SEQ ID NO. 9, an active fragment or analogue thereof, or a receptor-binding but otherwise inactive or partially inactive variant thereof.

A fourth aspect of the invention provides vectors comprising the cDNA of the invention or a nucleic acid according to the third aspect of the invention, and host cells transformed or transfected with nucleic acids or vectors of the invention. These cells are particularly suitable for expression of the polypeptide of the invention, and include insect cells such as Sf9 cells, obtainable from the American Type Culture Collection (ATCC SRL-171),

transformed with a baculovirus vector, and the human embryo kidney cell line 293EBNA transfected by a suitable expression plasmid. Preferred vectors of the invention are expression vectors in which a nucleic acid according to the invention is operatively connected to one or more appropriate promoters and/or other control sequences, such that appropriate host cells transformed or transfected with the vectors are capable of expressing the polypeptide of the invention. Other preferred vectors are those suitable for transfection of mammalian cells, or for gene therapy, such as adenovirus or retrovirus vectors or liposomes. A variety of such vectors is known in the art.

The invention also provides a method of making a vector capable of expressing a polypeptide encoded by a nucleic acid according to the invention, comprising the steps of operatively connecting the nucleic acid to one or more appropriate promoters and/or other control sequences, as described above.

The invention further provides a method of making a polypeptide according to the invention, comprising the steps of expressing a nucleic acid or vector of the invention in a host cell, and isolating the polypeptide from the host cell or from the host cell's growth medium. In one preferred embodiment of this aspect of the invention, the expression vector further comprises a sequence encoding an affinity tag, such as FLAG™ or hexahistidine, in order to facilitate purification of the polypeptide by affinity chromatography.

In yet a further aspect, the invention provides an antibody specifically reactive with a polypeptide of the invention. This aspect of the invention includes antibodies specific for the variant forms, fragments and analogues of VEGF-D referred to above. Such antibodies are useful as inhibitors or agonists of VEGF-D and as diagnostic agents for detection and quantification of VEGF-D. Polyclonal or monoclonal antibodies may be used. Monoclonal and

polyclonal antibodies can be raised against polypeptides of the invention using standard methods in the art. For some purposes, for example where a monoclonal antibody is to be used to inhibit effects of VEGF-D in a clinical situation,

5   it may be desirable to use humanized or chimeric monoclonal antibodies. Methods for producing these, including recombinant DNA methods, are also well known in the art.

This aspect of the invention also includes an antibody which recognises VEGF-D and which is suitably labelled.

10   Polypeptides or antibodies according to the invention may be labelled with a detectable label, and utilised for diagnostic purposes. Similarly, the thus-labelled polypeptide of the invention may be used to identify its corresponding receptor in situ. The polypeptide or antibody

15   may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, ecogenic or radioactive agent for imaging. For use in diagnostic assays, radioactive or non-radioactive labels, the latter including enzyme labels or labels of the biotin/avidin

20   system, may be used.

Clinical applications of the invention include diagnostic applications, acceleration of angiogenesis in wound healing, tissue or organ transplantation, or to establish collateral circulation in tissue infarction or

25   arterial stenosis, such as coronary artery disease, and inhibition of angiogenesis in the treatment of cancer or of diabetic retinopathy. Quantitation of VEGF-D in cancer biopsy specimens may be useful as an indicator of future metastatic risk.

30   Inasmuch as VEGF-D is highly expressed in the lung, and it also increases vascular permeability, it is relevant to a variety of lung conditions. VEGF-D assays could be used in the diagnosis of various lung disorders. VEGF-D could also be used in the treatment of lung disorders to improve

35   blood circulation in the lung and/or gaseous exchange between the lungs and the blood stream. Similarly, VEGF-D

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could be used to improve blood circulation to the heart and O_2 gas permeability in cases of cardiac insufficiency.   In like manner, VEGF-D could be used to improve blood flow and gaseous exhange in chronic obstructive airway disease.

Conversely, VEGF-D antagonists (e.g. antibodies and/or inhibitors) could be used to treat in conditions, such as congestive heart failure, involving accumulations of fluid in, for example, the lung resulting from increases in vascular permeability, by exerting an offsetting effect on vascular permeability in order to counteract the fluid accumulation.

VEGF-D is also expressed in the small intestine and colon, and administrations of VEGF-D could be used to treat malabsorptive syndromes in the intestinal tract as a result of its blood circulation increasing and vascular permeabiltiy increasing activities.

Thus the invention provides a method of stimulation of angiogenesis and/or neovascularization in a mammal in need of such treatment, comprising the step of administering an effective dose of VEGF-D, or a fragment or analogue thereof which has the ability to stimulate endothelial cell proliferation, to the mammal.

Optionally VEGF-D may be administered together with, or in conjunction with, one or more of VEGF-A, VEGF-B, VEGF-C, PlGF, PDGF, FGF and/or heparin.

Conversely the invention provides a method of inhibiting angiogenesis and/or neovascularization in a mammal in need of such treatment, comprising the step of administering an effective amount of an antagonist of VEGF-D to the mammal.   The antagonist may be any agent that prevents the action of VEGF-D, either by preventing the binding of VEGF-D to its corresponding receptor or the target cell, or by preventing activation of the transducer of the signal from the receptor to its cellular site of action.   Suitable antagonists include, but are not limited to, antibodies directed against VEGF-D; competitive or non-

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competitive inhibitors of binding of VEGF-D to the VEGF-D receptor, such as the receptor-binding but non-mitogenic VEGF-D variants referred to above; and anti-sense nucleotide sequences complementary to at least a part of the DNA

5   sequence encoding VEGF-D.

The invention also provides a method of detecting VEGF-D in a biological sample, comprising the step of contacting the sample with a reagent capable of binding VEGF-D, and detecting the binding. Preferably the reagent

10 capable of binding VEGF-D is an antibody directed against VEGF-D, more preferably a monoclonal antibody. In a preferred embodiment the binding and/or extent of binding is detected by means of a detectable label; suitable labels are discussed above.

15 Where VEGF-D or an antagonist is to be used for therapeutic purposes, the dose and route of application will depend upon the condition to be treated, and will be at the discretion of the attending physician or veterinarian. Suitable routes include subcutaneous, intramuscular or

20 intravenous injection, topical application, implants etc. Topical application of VEGF-D may be used in a manner analogous to VEGF.

According to yet a further aspect, the invention provides diagnostic/prognostic means typically in the form

25 of test kits. For example, in one embodiment of the invention there is provided a diagnostic/prognostic test kit comprising an antibody to VEGF-D and means for detecting, and more preferably evaluating, binding between the antibody and VEGF-D. In one preferred embodiment of the

30 diagnostic/prognostic means according to the invention, either the antibody or the VEGF-D is labelled with a detectable label, and either the antibody or the VEGF-D is substrate-bound, such that the VEGF-D-antibody interaction can be established by determining the amount of label

35 attached to the substrate following binding between the antibody and the VEGF-D. In a particularly preferred

embodiment of the invention, the diagnostic/prognostic means may be provided as a conventional ELISA kit.

In another alternative embodiment, the diagnostic/prognostic means may comprise polymerase chain reaction means for establishing the genomic sequence structure of a VEGF-D gene of a test individual and comparing this sequence structure with that disclosed in this application in order to detect any abnormalities, with a view to establishing whether any aberrations in VEGF-D expression are related to a given disease condition.

In accordance with a further aspect, the invention relates to a method of detecting aberrations in VEGF-D gene structure in a test subject which may be associated with a disease condition in said test subject. This method comprises providing a DNA sample from said test subject; contacting the DNA sample with a set of primers specific to VEGF-D DNA operatively coupled to a polymerase and selectively amplifying VEGF-D DNA from the sample by polymerase chain reaction, and comparing the nucleotide sequence of the amplified VEGF—D DNA from the sample with the nucleotide sequences set forth in SEQ ID NO:1 or SEQ ID NO:4. The invention also includes the provision of a test kit comprising a pair of primers specific to VEGF-D DNA operatively coupled to a polymerase, whereby said polymerase is enabled to selectively amplify VEGF-D DNA from a DNA sample.

Another aspect of the invention concerns the provision of a pharmaceutical composition comprising either VEGF-D polypeptide or a fragment or analogue thereof which promotes proliferation of endothelial cells, or an antibody thereto. Compositions which comprise VEGF-D polypeptide may optionally further comprise one or more of VEGF, VEGF-B and VEGF-C, and/or heparin.

In another aspect, the invention relates to a protein dimer comprising VEGF-D polypeptide, particularly a disulphide-linked dimer. The protein dimers of the

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invention include both homodimers of VEGF-D polypeptide and
heterodimers of VEGF-D and VEGF, VEGF-B, VEGF-C, PlGF or
PDGF.

According to a yet further aspect of the invention
there is provided a method for isolation of VEGF-D
comprising the step of exposing a cell which expresses
VEGF-D to heparin to facilitate release of VEGF-D from the
cell, and purifying the thus-released VEGF-D.

Another aspect of the invention involves providing a
vector comprising an anti-sense nucleotide sequence which is
complementary to at least a part of a DNA sequence which
encodes VEGF-D or a fragment or analogue thereof which
promotes proliferation of endothelial cells. According to
a yet further aspect of the invention such a vector
comprising an anti-sense sequence may be used to inhibit, or
at least mitigate, VEGF-D expression. The use of a vector
of this type to inhibit VEGF-D expression is favoured in
instances where VEGF-D expression is associated with a
disease, for example where tumours produce VEGF-D in order
to provide for angiogenesis. Transformation of such tumour
cells with a vector containing an anti-sense nucleotide
sequence would suppress or retard angiogenesis, and so would
inhibit or retard growth of the tumour.

Polynucleotides of the invention such as those
described above, fragments of those polynucleotides, and
variants of those polynucleotides with sufficient similarity
to the non-coding strand of those polynucleotides to
hybridise thereto under stringent conditions all are useful
for identifying, purifying, and isolating polynucleotides
encoding other, non-human, mammalian forms of VEGF-D. Thus,
such polynucleotide fragments and variants are intended as
aspects of the invention. Exemplary stringent hybridisation
conditions are as follows: hybridisation at 42°C in 5X SSC,
20 mM NaPO$_4$, pH 6.8, 50% formamide; and washing at 42°C in
0.2X SSC. Those skilled in the art understand that it is
desirable to vary these conditions empirically based on the

length and the GC nucleotide base content of the sequences to be hybridised, and that formulae for determining such variation exist. See for example Sambrook *et al*, "Molecular Cloning: A Laboratory Manual", Second Edition, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1989).

Moreover, purified and isolated polynucleotides encoding other, non-human, mammalian VEGF-D forms also are aspects of the invention, as are the polypeptides encoded thereby, and antibodies that are specifically immunoreactive with the non-human VEGF-D variants. Thus, the invention includes a purified and isolated mammalian VEGF-D polypeptide, and also a purified and isolated polynucleotide encoding such a polypeptide.

It will be clearly understood that nucleic acids and polypeptides of the invention may be prepared by synthetic means or by recombinant means, or may be purified from natural sources.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a comparison between the sequences of human VEGF-D and human VEGF$_{165}$ (Figure 1a), human VEGF-B (Figure 1b), human VEGF-C (Figure 1c) and human PlGF (Figure 1d). The box indicates residues which match those in human VEGF-D exactly;

Figure 2 shows sequence alignments between the sequences of human VEGF-D, human VEGF$_{165}$, human VEGF-B, human VEGF-C and human PlGF. The boxes indicate residues that match the VEGF-D sequence exactly; and

Figure 3 shows the amino acid sequence of human VEGF-D (SEQ ID NO 3), as predicted from the cDNA sequence (SEQ ID NO 1). The boxes indicate potential sites for N-linked glycosylation.

Figure 4 shows the nucleotide sequence of a second cDNA sequence encoding human VEGF-D (SEQ ID NO 4), isolated by hybridisation from a commercial human lung cDNA library; this cDNA contains the entire coding region for human VEGF-D;

Figure 5 shows the amino acid sequence for human VEGF-D (SEQ ID NO 5) deduced from the sequence of the cDNA of Figure 4;

Figure 6 shows the nucleotide sequence of cDNA encoding mouse VEGF-D1 (SEQ ID NO 6), isolated by hybridisation screening for a commercially-available mouse lung cDNA library;

Figure 7 shows the nucleotide sequence of cDNA encoding mouse VEGF-D2 (SEQ ID NO 7), isolated from the same library as in Figure 6;

Figure 8 shows the deduced amino acid sequences for mouse VEGF-D1 (SEQ ID NO 8) and VEGF-D2 (SEQ ID NO 9);

Figure 9 shows a comparison between the deduced amino acid sequences of mouse VEGF-D1, mouse VEGF-D2 and human VEGF-D;

Figure 10 shows sequence alignments between the amino acid sequences of human VEGF-D, human $VEGF_{165}$, human VEGF-B, human VEGF-C and human PlGF; and

Figure 11 shows the results of a bioassay in which conditioned medium from COS cells expressing either VEGF-A or VEGF-D was tested for ability to bind to the extracellular domain of a chimeric receptor expressed in Ba/F3 cells.

Figure 12 shows the results of immunoprecipitation and Western blotting analysis of VEGF-D peptides

(A)   pEFBOSVEGFDfullFLAG and pCDNA-1VEGF-A were transfected into COS cells and biosynthetically labelled with ^{35}S-cysteine/methionine for 4 hours. The supernatants from these cultures were immunoprecipitated with either M2 gel or an antiserum directed to VEGF-A coupled to protein A. Washed beads were eluted with an equal volume of 2 x SDS-PAGE sample buffer and boiled. The samples were then resolved by 12% SDS-PAGE. Lanes marked with an asterix (*) indicate where samples were reduced with dithiothreitol and alkylated with iodoacetamide. Molecular weight markers are indicated. fA and fB indicate the 43 kD and 25 kD species

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immunoprecipitated by the M2 gel from the COS cells expressing pEFBOSVEGFDfullFLAG.

(B)   Western blotting analysis of purified VEGFDΔNΔC. An aliquot of material eluted from the M2 affinity column
5   (fraction #3, VEGFDΔNΔC) was combined with 2 x SDS-PAGE sample buffer and resolved on a 15% SDS-PAGE gel.   The proteins were then transferred to nitrocellulose membrane and probed with either monoclonal antibody M2 or a control isotype-matched antibody (Neg).   Blots were developed using
10 a   goat   anti-mouse-HRP   secondary   antibody   and chemiluminescence (ECL , Amersham).   Monomeric VEGFDΔNΔC is arrowed, as is the putative dimeric form of this peptide (VEGFDΔNΔC").   Molecular weight markers are indicated.

Figure 13 shows the results of analysis of VEGFDΔNΔC
15 protein using the VEGFR2 bioassay.   Recombinant VEGFDΔNΔC, and material purified by M2 affinity chromatography, was assessed using the VEGFR2 bioassay. Bioassay cells (10^4), washed to remove IL-3, were incubated with aliquots of conditioned medium from VEGF-D transfected COS cells,
20 fraction #1 from the affinity column (void volume) or fraction #3 from the affinity column (containing VEGFDΔNΔC). All samples were tested at an initial concentration of 20% (ie 1/5) followed by doubling dilutions.   Cells were allowed to incubate for 48 hours at 37°C in a humidified atmosphere
25 of 10% CO$_2$.   Cell proliferation was quantitated by the addition of 1 μCi of ^{3}H-thymidine and counting the amount incorporated over a period of 4 hours.

Figure 14 shows stimulation of tyrosine phosphorylation of the VEGFR3 receptor (Flt4) on NIH3T3 cells by culture
30 supernatant from HF cells infected with a recombinant baculovirus vector transformed with VEGF-D.

Figure 15 shows stimulation of tyrosine phosphorylation of the VEGFR2 receptor (KDR) in PAE cells by culture supernatant prepared as in Figure 14.

35 Figure 16 shows the mitogenic effect of VEGFDΔNΔC on bovine aortic endothelial cells (BAEs).   BAEs were treated

with fraction #3 containing VEGFDΔNΔC and, as positive
control, purified VEGF-A as described in the text. The
result obtained using medium without added growth factor is
denoted Medium Control.

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DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by
reference to the figures, and to the following non-limiting
examples.

10

Example 1

It has been speculated that no further members of the
VEGF family will be found, because there are no known orphan
receptors in the VEGFR family. Furthermore, we are not
15   aware of any suggestion in the prior art that other such
family members would exist.

A computer search of nucleic acid databases was carried
out incidentally to another project, using as search topics
the amino acid sequences of VEGF, VEGF-B, VEGF-C and PlGF.
20   Several cDNA sequences were identified by this search. One
of these sequences, GenBank Accession No. H24828, encoded a
polypeptide which was similar in structure to the cysteine-
riched C-terminal region of VEGF-C. This sequence was
obtained from the database of expressed sequence tags
25   (dbEST), and for the purposes of this specification is
designated XPT. The XPT cDNA had been isolated from a human
cDNA library designated "Soares Breast 3NbHBst", which was
constructed using mRNA from an adult human female breast
tissue. As far as can be ascertained this was normal breast
30   tissue. Sequencing of the XPT DNA was performed pursuant to
the Integrated Molecular Analysis of Genome Expression
Consortium (IMAGE Consortium), which solicits cDNA libraries
from laboratories around the world, arrays the cDNA clones,
and provides them to other organisations for sequencing.
35   The XPT sequence shown in the database was 419
nucleotides long, and encoded an amino acid sequence similar

to the C-terminal 100 amino acids of VEGF-C, *ie.*
approximately residues 250 to 350, using the numbering
system of Joukov *et al* (1996). Similarly cysteine-rich
regions are found in other proteins, which are entirely
5   unrelated in function to the VEGF family, for example the
secreted silk-like protein sp185 synthesized in the salivary
glands of the midge *Chironomus tentans*. This protein is
encoded by the gene BR3, located in a Balbiani ring, a
tissue specific chromosome "puff" found on polytene
10   chromosomes in the midge salivary gland (Dignam and Case:
Gene, 1990 88 133-140; Paulsson *et al*, J. Mol. Biol., 1990
211 331-349). It is stated in Joukov *et al* (1996) that the
sp185-like structural motif in VEGF-C may fold into an
independent domain, which is thought to be at least
15   partially cleaved off after biosynthesis, and that there is
at least one cysteine motif of the sp185 type in the
C-terminal region of VEGF.

Figure 3 of Joukov *et al* shows that the last two-thirds
of the C-terminal cysteine-rich region of VEGF-C do not
20   align with VEGF or PlGF, and in fact could be considered a
C-terminal extension of VEGF-C which is not present in VEGF
or PlGF. The sequence encoded by XPT is similar to this
extension. As the XPT cDNA was truncated at its 5' end, it
was not possible to deduce or predict any amino acid
25   sequence for regions N-terminal to the cysteine-rich domain.
Thus the portion of VEGF-C which is similar to the XPT-
derived sequence does not extend to regions of VEGF-C which
are conserved among other members of the VEGF family.

As described above, it was not possible to predict
30   whether the N-terminal region of the polypeptide encoded by
a full-length XPT nucleic acid (as distinct from the
truncated XPT cDNA reported in dbEST) would show any further
homology to any member of the VEGF family, in particular
VEGF-C, which has a further N-terminal 250 amino acids. For
35   example, the naturally-occurring protein encoded by a full-
length XPT nucleic acid could have been the human homologue

of the midge salivary gland protein.   Alternatively, the type of cysteine-rich motif encoded by truncated XPT cDNA could be widely distributed among proteins, as are many structural domains.   For example, clusters of cysteine

5   residues may be involved in metal binding, formation of intramolecular disulphide bonds to promote accurate protein folding, or formation of intermolecular disulphide bonds for assembly of protein subunits into complexes (Dignam and Chase, 1990).   In order to determine whether the truncated

10 XPT cDNA was derived from sequences encoding a VEGF-related molecule, it was necessary to isolate a much longer cDNA.

Example 2   Cloning of cDNA Encoding VEGF-D

        A sample of the XPT cDNA reported in dbEST was

15 obtained from the American Type Culture Collection, which is a registered supplier of cDNA clones obtained by the IMAGE Consortium.   The identity of the XPT cDNA was confirmed by nucleotide sequencing, using the dideoxy chain termination method (Sanger et al, Proc. Natl. Acad. Sci. USA, 1977 74

20 5463-5467).

        The XPT cDNA was used as a hybridisation probe to screen a human breast cDNA library, which was obtained commercially from Clontech.   One positive clone was isolated, and this clone was then sequenced on both strands.

25 The nucleotide sequence was compiled, and an open reading frame was identified.   The nucleic acid sequence is set out in SEQ ID NO. 1.   The polypeptide encoded by this sequence was designated VEGF-D, and its deduced amino acid sequence, designated SEQ ID NO. 3, is set out in Figure 3.   In Figure

30 3 putative sites of N-linked glycosylation, with the consensus sequence N-X-S/T in which X is any amino acid, are indicated by the boxes.

Example 3   Characteristics of VEGF-D

35   The amino acid sequence of VEGF-D was compared with those of human VEGF-A$_{165}$, VEGF-B, VEGF-C and PlGF.   These

- 20 -

comparisons are set out in Figures 1a to d respectively. The degree of sequence homology was calculated, and if gaps in sequence introduced for the purposes of alignment are not considered in the calculation, VEGF-D is 31% identical to

5   VEGF, 48% identical to VEGF-C, 28% identical to VEGF-B and 32% identical to PlGF. Thus the most closely-related protein identified was VEGF-C.

Computer searches of the GenBank, EMBL and SwissProt nucleic acid databases did not reveal any protein sequences

10   identical to VEGF-D. As expected from the sequence alignment referred to above, the most closely related protein found in these databases was VEGF-C. Searches of dbEST were also performed, but did not reveal any sequences encompassing the entire coding region of VEGF-D. The

15   sequence of VEGF-D is unrelated to that of Tie-2 ligand 1 as disclosed in WO 96/11269.

It is important to bear in mind that the only homologies detected were at the level of amino acid sequence. Thus it would not have been possible to isolate

20   the cDNA or gDNA encoding VEGF-D by methods such as low-stringency hybridization with a nucleic acid sequence encoding another member of the VEGF family.

VEGF-D appears to be most closely related to VEGF-C of all the members of the VEGF family. Because the VEGF-D

25   amino acid sequence includes the cysteine-rich sp185-like motif which is found in VEGF-C, the polypeptide of the invention may play an important functional role in lymphatic endothelia. While we do not wish to be bound by any proposed mechanism, it is thought that VEGF-C and VEGF-D may

30   constitute a silk-like matrix over which endothelial cells can grow. Lymphatic vessels have no basement membrane, so the silk-like matrix can form a basement membrane-like material. This may be important in promoting cell growth and/or in cell differentiation, and may be relevant to

35   cancer, especially metastasis, drug therapy, cancer prognosis, etc.

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Example 4   Biological Characteristics of VEGF-D

       The cDNA sequence of VEGF-D was used to predict the deduced amino acid sequence of VEGF-D, the biochemical characteristics of the encoded polypeptide, including the numbers of strongly basic, strongly acidic, hydrophobic and polar amino acids, the molecular weight, the isoelectric point, the charge at pH 7, and the compositional analysis of the whole protein. This analysis was performed using the Protean protein analysis program, Version 1.20 (DATASTAR). These results are summarised in Tables 1 and 2 below. Table 1 also shows the codon usage.

Table 1

Translated DNA Sequence of VEGF-D contig x(1,978)
With Standard Genetic Code

Molecular Weight 37056.60 Daltons

425 Amino Acids

46 Strong Basic(+) Amino Acids (K,R)

41 Strong Acidic(-) Amino Acids (D,E)

79 hydrophobic Amino Acids (A,I,L,F,W,V)

108 Polar Amino Acids (N,C,Q,S,T,Y)

7.792 Isoelectric Point

6.371 Charge at pH 7.0

Total number of bases translated is 978

% A = 28.73 [281]

% G = 23.11 [226]

% T = 23.21 [227]

% C = 24.95 [244]

% Ambiguous = 0.00   [0]

% A+T = 51.94   [508]

% C+G = 48.06   [470]

Davis, Botstein, Roth Melting Temp °C.   84.09

Wallace Temp °C.   3384.00

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Table 1 (cont.)

Codon usage:							
ccg ( )	0 #	ugc Cys(C)	14 #	cuc Leu(L)	6 #	ucg Ser(S)	
uaa ( )	0 #	ugu Cys(C)	16 #	cug Leu(L)	4 #	ucu Ser(S)	
uag ( )	0 #	--- Cys(C)	30 #	cuu Leu(L)	2 #	--- Ser(S)	3
--- ( )	0 #	caa Gln(Q)	1 #	uua Leu(L)	1 #	uga Ter(.)	
gca Ala(A)	5 #	cag Gln(Q)	11 #	uug Leu(L)	5 #	--- Ter(.)	
gcc Ala(A)	4 #	--- Gln(Q)	12 #	--- Leu(L)	23 #	aca Thr(T)	
gcg Ala(A)	1 #	gaa Glu(E)	16 #	aaa Lys(K)	13 #	acc Thr(T)	
gcu Ala(A)	5 #	gag Glu(E)	12 #	aag Lys(K)	10 #	acg Thr(T)	
--- Ala(A)	15 #	--- Glu(E)	28 #	--- Lys(K)	23 #	acu Thr(T)	
aga Arg(R)	7 #	gga Gly(G)	1 #	aug Met(M)	6 #	--- Thr(T)	2
agg Arg(R)	5 #	ggc Gly(G)	2 #	--- Met(M)	6 #	ugg Trp(W)	
cga Arg(R)	5 #	ggg Gly(G)	3 #	uuc Phe(F)	4 #	--- Trp(W)	
cgc Arg(R)	4 #	ggu Gly(G)	2 #	uuu Phe(F)	8 #	uac Tyr(Y)	
cgg Arg(R)	1 #	--- Gly(G)	8 #	--- Phe(F)	12 #	uau Tyr(Y)	
cgu Arg(R)	1 #	cac His(H)	7 #	cca Pro(P)	9 #	--- Tyr(Y)	
--- Arg(R)	23 #	cau His(H)	7 #	ccc Pro(P)	6 #	gua Val(V)	
aac Asn(N)	5 #	--- His(H)	14 #	ccu Pro(P)	8 #	guc Val(V)	
aau Asn(N)	4 #	aua Ile(I)	2 #	--- Pro(P)	23 #	gug Val(V)	
--- Asn(N)	9 #	auc Ile(I)	6 #	agc Ser(S)	6 #	guu Val(V)	
gac Asp(D)	8 #	auu Ile(I)	5 #	agu Ser(S)	8 #	--- Val(V)	1
qau Asp(D)	5 #	--- Ile(I)	13 #	uca Ser(S)	5 #	nnn ???(X)	
gau Asp(D)	5 #	--- Ile(I)	13 #	uca Ser(S)	5 #	nnn ???(X)	
--- Asp(D)	13 #	cua Leu(L)	5 #	ucc Ser(S)	7 #	TOTAL	32

Contig 2:

Contig Length:	2379 bases
Average Length/Sequence:	354 bases
Total Sequence Length:	4969 bases

Table 2

Predicted Structural Class of the Whole Protein:

Deléage & Roux Modification of Nishikawa & Ooi 1987

Analysis	Whole Protein
Molecular Weight	37056.60 m.w.
Length	325
1 microgram =	26.986 pMoles
Molar Extinction coefficient	30200±5%
1 A(280) =	1.23 mg/ml
Isoelectric Point	7.79
Charge at pH 7	6.37

Table 2 (cont.)

Whole Protein Composition Anayalsis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	134	46.30	41.23
Acidic (DE)	41	13.79	12.62
Basic (KR)	46	17.65	14.15
Polar (NCQSTY)	108	30.08	33.23
Hydrophobic (AILFWV)	79	23.86	24.31
A Ala	15	2.88	4.62
C Cys	30	8.35	9.23
D Asp	13	4.04	4.00
E Glu	28	9.75	8.62
F Phe	12	4.77	3.69
G Gly	8	1.23	2.46
H His	14	5.18	4.31
I Ile	13	3.97	4.00
K Lys	23	7.96	7.08
L Leu	23	7.03	7.08
M Met	6	2.12	1.85
N Asn	9	2.77	2.77
P Pro	23	6.08	7.08
Q Gln	12	4.15	3.69
R Arg	23	9.69	7.08
S Ser	33	7.76	10.15
T Thr	21	5.73	6.46
V Val	12	3.21	3.69
W Trp	4	2.01	1.23
Y Trp	3	1.32	0.92
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	0	0.00	0.00

This analysis predicts a molecular weight for the unprocessed VEGF-D monomer of 37 kilodaltons (kD), compared to the experimentally determined values (for the fully processes peptides) of 20 to 27 kD for VEGF-A monomers, 21 kD for the VEGF-B monomer and 23 kD for the VEGF-C monomer.

Example 5

The original isolation of a cDNA for VEGF-D, described in Example 2 involved hybridisation screening of a human breast cDNA library. As only one cDNA clone for VEGF-D was thus isolated, it was not possible to confirm the structure of the cDNA by comparison with other independently isolated VEGF-D cDNAs. The work described in this example, which involved isolation of additional human VEGF-D cDNA clones, was carried out in order to confirm the structure of human VEGF-D cDNA. In addition, mouse VEGF-D cDNA clones were isolated.

Two cDNA libraries which had been obtained commercially from Stratagene, one for human lung and one for mouse lung (catalogue numbers 937210 and 936307, respectively) were used for hybridisation screening with a VEGF-D cDNA probe. The probe, which spanned from nucleotides 1817 to 2495 of the cDNA for human VEGF-D described in Example 2, was generated by polymerase chain reaction (PCR) using a plasmid containing the VEGF-D cDNA as template and the following two oligonucleotides:

5'-GGGCTGCTTCTAGTTTGGAG (SEQ ID NO. 10), and

5'-CACTCGCAACGATCTTCGTC (SEQ ID NO. 11).

Approximately two million recombinant bacteriophage were screened with this probe from each of the two cDNA libraries. Nine human and six mouse cDNA clones for VEGF-D were subsequently isolated.

Two of the nine human cDNA clones for VEGF-D were sequenced completely using the dideoxy chain termination method (Sanger et al, Proc. Natl. Acad. Sci. USA, 1977 74

5463-5467). The two cDNAs contained the entire coding
region for human VEGF-D, and were identical except that
one of the clones was five nucleotides longer than the
other at the 5'-terminus. The nucleotide sequence of the
5   shorter cDNA is shown in Figure 4, and is designated SEQ
ID NO. 4. The amino acid sequence for human VEGF-D
(hVEGF-D) deduced from this cDNA was 354 residues long,
and is shown in Figure 5; this is designated SEQ ID NO. 5.
The sequences of the 5' regions of five of the other human
10 VEGF-D cDNA clones were also determined. For each clone,
the sequence that was characterized contained more than
100 nucleotides of DNA immediately downstream from the
translation start site of the coding region. In all
cases, the sequences of these regions were identical to
15 corresponding regions of the human VEGF-D cDNA shown in
Figure 4.

All six mouse cDNA clones for VEGF-D were
sequenced completely. Only two of the clones contained an
entire coding region for VEGF-D; the other clones were
20 truncated. The nucleotide sequences of the two clones
with an entire coding region are different, and encode
amino acid sequences of different sizes. The longer amino
acid sequence is designated mVEGF-D1, and the shorter
sequence is designated mVEGF-D2. The nucleotide sequences
25 of the cDNAs encoding mVEGF-D1 and mVEGF-D2 are shown in
Figures 6 and 7 respectively. The deduced amino acid
sequences for mVEGF-D1 and mVEGF-D2 are shown in Figure 8.
These sequences are respectively designated SEQ ID NOS. 6,
7, 8 and 9. The differences between the amino acid
30 sequences are:

      i) an insertion of five amino acids (DFSFE) after
residue 30 in mVEGF-D1 in comparison to mVEGF-D2;

      ii) complete divergence of the C-terminal ends
after residue 317 in mVEGF-D1 and residue 312 in mVEGF-D2,
35 which results in mVEGF-D1 being considerably longer.

Three of the four truncated cDNAs for mouse VEGF-D encoded the C-terminal region, but not the N-terminal 50 amino acids. All three of these cDNAs encoded a C-terminal end for VEGF-D which is identical to that for

5   mVEGF-D2. The other truncated cDNA encoded only the N-terminal half of VEGF-D. The amino acid sequence deduced from this cDNA contained the five amino acids DFSFE immediately after residue 30 found in mVEGF-D1, but not in mVEGF-D2.

10 As described above, the entire sequence of the human VEGF-D cDNA clone reported in this example has been validated by comparison with that for a second human clone. In addition, the sequence of the 5' end of the coding region was found to be identical in five other

15 human VEGF-D cDNA clones. In contrast, the sequence reported in Example 2 contained most of the coding region for VEGF-D, but was incorrect near the 5'-end of this region. This was probably because the VEGF-D cDNA was truncated near the 5'-end of the coding region and at that

20 point had been ligated with another unidentified cDNA, and consequently the first 30 codons of the true coding sequence for VEGF-D had been deleted and replaced with a methionine residue. This methionine residue was defined as the N-terminal amino acid of the VEGF-D sequence

25 presented in Example 2.

The N-terminal regions of the deduced amino acid sequences of mouse VEGF-D1 and VEGF-D2 are very similar to that deduced for human VEGF-D (see Figure 9). This also indicates that the correct deduced amino acid sequence for

30 human VEGF-D is reported in this example. The N-terminal 25 amino acids of human VEGF-D form an extremely hydrophobic region, which is consistent with the notion that part of this region may be a signal sequence for protein secretion. Figure 10 shows the alignment of the

35 human VEGF-D sequence with the sequences of other members of the VEGF family of growth factors, namely human $VEGF_{165}$

(hVEGF$_{165}$), human VEGF-B (hVEGF-B), human VEGF-C (hVEGF-C) and human Placental Growth Factor (hPlGF). When gaps in the alignments are ignored for the purposes of calculation, human VEGF-D is found to be 31% identical in amino acid sequence to human VEGF$_{165}$, 28% identical to human VEGF-B, 48% identical to VEGF-C and 32% identical to human PlGF. Clearly VEGF-C is the member of this family which is most closely related to VEGF-D.

The differences in sequence for mouse VEGF-D1 and VEGF-D2 most probably arise from differential mRNA splicing. The C-terminal 41 amino acid residues of VEGF-D1 are deleted in VEGF-D2, and are replaced with 9 residues which are not closely related to the VEGF-D1 sequence. Therefore 4 cysteine residues present near the C-terminus of VEGF-D1 are deleted in VEGF-D2. This change may alter the tertiary or quaternary structures of the protein, or may affect the localisation of the protein in the cell or the extracellular environment. The C-terminal end of human VEGF-D resembles that of mouse VEGF-D1, not mouse VEGF-D2. The small 5 amino acid insertion after residue 30 in mouse VEGF-D1, which is not present in either mouse VEGF-D2 or human VEGF-D, may influence proteolytic processing of the protein.

VEGF-D is highly conserved between mouse and man. Eighty-five percent of the amino acid residues of human VEGF-D are identical in mouse VEGF-D1. This is likely to reflect conservation of protein function. Putative functions for VEGF-D have been proposed herein. Although we have not found alternative forms of human VEGF-D cDNA, it is possible that the RNA splice variation which gives rise to numerous forms of mRNA for mouse VEGF-D may also occur in human tissues.

Example 6   Expression of VEGF-D in COS Cells

A fragment of the human cDNA for VEGF-D, spanning from nucleotide 1 to 1520 of the sequence shown in Figure

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4 and containing the entire coding region, was inserted into the mammalian expression vector pCDNA1-amp. The vector was used to transiently transfect COS cells by the DEAE-Dextran method as described previously (Aruffo and

5   Seed, 1987) and the resulting conditioned cell culture media, collected after 7 days of incubation, were concentrated using Amicon concentrators (Centricon 10 with a 10,000 molecular weight cut off) according to the manufacturer. The plasmids used for transfections were

10   the expression construct for human VEGF-D and, as positive control, a construct made by insertion of mouse VEGF-A cDNA into pCDNA1-amp. The conditioned media were tested in two different bioassays, as described below, and the results demonstrate that the COS cells did in fact express

15   and secrete biologically-active VEGF-D.

Example 7   Bioassay for Capacity of VEGF-D to Bind to
             VEGF Receptor-2

             As shown in Example 5, VEGF-D is closely related

20   in primary structure to other members of the VEGF family. Most members of this protein family are mitogenic and/or chemotactic for endothelial cells (Keck et al, 1989; Leung et al, 1989; Joukov, et al, 1996; Olofsson et al, 1996). In addition VEGF-A (previously known as VEGF), the first

25   member of the VEGF family to be described in the literature, is a potent inducer of vascular permeability (Keck et al, 1989). As protein structure is an important determinant of protein function, it seemed likely that VEGF-D might also be mitogenic for endothelial cells or

30   induce vascular permeability. Therefore human VEGF-D was tested in a bioassay for its capacity to bind to VEGF receptor-2 (VEGFR2; also known as Flk-1), an endothelial cell-specific receptor which, when activated by VEGF-A, is thought to give rise to a mitogenic signal (Strawn et al,

35   1996).

A bioassay for detection of growth factors which bind to VEGFR2 has been developed in the factor-dependent cell line Ba/F3, and is described in our earlier patent application, No. PCT/US95/16755. These cells grow in the

5   presence of interleukin-3 (IL-3); however removal of this factor results in cell death within 48 hours. If another receptor capable of delivering a growth stimulus is transfected into the Ba/F3 cells, the cells can be rescued by the specific growth factor which activates that

10   receptor when the cells are grown in medium lacking IL-3. In the specific case of receptor-type tyrosine kinases (eg. VEGFR2), chimeric receptors containing the extracellular domain of the receptor tyrosine kinase and the transmembrane and cytoplasmic domains of the

15   erythropoietin receptor (EpoR) can be utilised. In this case stimulation with the ligand (eg. VEGF), which binds to the extracellular domain of the chimeric receptor, results in signalling via the EpoR cytoplasmic domain and subsequent rescue of the cell line in growth medium

20   lacking IL-3. The construction of the chimeric receptor used in this study, consisting of the mouse VEGFR2 extracellular domain and the mouse EpoR transmembrane and cytoplasmic domains, and the bioassay itself are described below.

25

Plasmid Construction

i)   Construction of a plasmid for generating chimeric VEGFR2 receptors

         To obtain a plasmid construct with which DNA

30   encoding the extracellular domain of mouse VEGFR2 could easily be ligated with DNA encoding other protein domains, site-directed mutagenesis was used to generate a BglII restriction enzyme site at the position of mouse VEGFR2 cDNA which encoded the junction of the extracellular

35   domain and the transmembrane domain. The full-length clone of the mouse VEGFR2 cDNA described by Oelrichs *et al*

(1993) was subcloned into the mammalian expression vector
pCDNA1-amp, using the BstXI restriction enzyme site.
Single stranded UTP+ DNA was generated using the M13
origin of replication, and this was used as a template to

5   generate mouse VEGFR2 cDNA containing the BglII site at
the desired position. The plasmid containing the altered
VEGFR2 cDNA was designated pVEGFR2Bgl. DNA fragments
encoding the transmembrane and cytoplasmic domains of any
receptor can be inserted at the BglII site of pVEGFR2Bgl

10   in order to generate chimeric VEGFR2 receptors.

ii)   Construction of VEGFR2/EpoR chimeric receptor
The mouse EpoR cDNA was subcloned into the
expression vector pCDNA1-amp, and single stranded DNA was

15   generated as a template for mutagenesis. A BglII
restriction enzyme site was inserted into the EpoR cDNA at
the position encoding the junction of the transmembrane
and extracellular domains of the EpoR to allow direct
ligation of this DNA fragment to the modified cDNA

20   encoding the extracellular domain of VEGFR2 in pVEGFR2Bgl.
In addition a BglII site in the cytoplasmic domain of the
EpoR was removed by a silent single nucleotide
substitution. The DNA fragment encoding the transmembrane
and cytoplasmic domains of EpoR was then used to replace

25   the portion of pVEGFR2Bgl encoding the transmembrane and
cytoplasmic domains of VEGFR2. Thus a single reading
frame was generated which encoded the chimeric receptor
consisting of the VEGFR2 extracellular domain and the EpoR
transmembrane and cytoplasmic domains.

30   The DNA fragment encoding the chimeric receptor
was subcloned into the expression vector pBOS, and co-
transfected into the Ba/F3 cell line with plasmid pgk-neo
at a ratio of 1:20. Cells expressing the VEGFR2-EpoR
protein were selected by flow cytometry analysis using a

35   monoclonal antibody to the VEGFR2 extracellular domain
(MAb 4H3). This monoclonal antibody is described in

Australian Patent Application No. PM 3794 filed 10
February 1994. Cell lines expressing higher levels of
VEGFR2-EpoR were selected by growing the cells in 5 μg/ml
MAb 4H3 or 25 ng/ml of recombinant VEGF. A cell line
5   expressing high levels of VEGFR2-EpoR, designated Ba/F3-
NYK-EpoR, was used for the bioassay.

The Bioassay

The Ba/F3-NYK-EpoR cells described above were
10   washed three times in PBS to remove all IL-3 and
resuspended at a concentration of 1000 cells per 13.5 μl
of culture medium and 13.5 μl was aliquoted per well of a
60-well Terasaki plate. Conditioned media from
transfected COS cells were then diluted into the cell
15   culture medium. Cells expressing a chimeric receptor
consisting of the extracellular domain of the endothelial
cell receptor Tie2 and the transmembrane and cytoplasmic
domains of EpoR were used as a non-responding control cell
line. Cells were incubated for 48-96 hours, during which
20   the cells incubated in cell culture medium alone had died
and the relative survival/proliferation seen in the other
wells (ie. in the presence of COS cell-conditioned media)
was scored by counting the viable cells present per well.
The conditioned medium from COS cells which had
25   been transiently transfected with expression plasmids was
concentrated 30-fold and used in the VEGFR2 bioassay.
Concentrated conditioned medium from COS cells transfected
with pCDNA1-amp was used as negative control.
The results are shown in Figure 11, with the
30   percentage of 30-fold concentrated COS cell-conditioned
medium in the incubation medium (vol/vol) plotted versus
the number of viable cells in the well after 48 hours of
incubation. Clearly the conditioned medium containing
either VEGF-A or VEGF-D was capable of promoting cell
35   survival in this assay, indicating that both proteins can
bind to and activate VEGFR2.

Example 8   Vascular Permeability Assay

     Human VEGF-D, prepared as in Example 6 and
concentrated 30-fold, was tested in the Miles vascular
permeability assay (Miles and Miles, 1952) performed in
5   anaesthetized guinea pigs (albino/white, 300-400 g).
Concentrated conditioned medium for COS cells transfected
with pCDNA1-amp was again used as a negative control.
Guinea pigs were anaesthetised with chloral-hydrate
(3.6 g/100 ml; 0.1 ml per 10 g of body weight). The backs
10   of the animals were then carefully shaved with clippers.
Animals were given an intracardiac injection of Evans Blue
dye (0.5% in MT PBS, 0.5 ml) using a 23G needle, and were
then injected intra-dermally with 100-150 μl of
concentrated COS cell-conditioned medium. After 15-20 min
15   the animals were sacrificed and the layer of skin on the
back excised to expose the underlying blood vessels.   For
quantitation, the area of each injection was excised and
heated to 45°C in 2-5 ml of formamide. The resulting
supernatants, containing extravasated dye, were then
20   examined spectrophotometrically at 620 nm.

    For animal 1, the absorbance at 620 nm arising
from injection of 30-fold concentrated VEGF-A conditioned
medium was 0.178, that for the 30-fold concentrated VEGF-D
conditioned medium was 0.114, and that for 30-fold
25   concentrated medium from cells transfected with pCDNA1-amp
was 0.004. For animal 2, the 30-fold concentrated media
were diluted 4-fold in cell culture medium before intra-
dermal injection. The absorbance at 620 nm for the VEGF-A
conditioned sample was 0.141, that for the VEGF-D
30   conditioned sample was 0.116 and that for a sample matched
for serum content as negative control was 0.017. The
enhanced extravasation of dye observed for both animals in
the presence of VEGF-A or VEGF-D demonstrated that both of
these proteins strongly induced vascular permeability.

35   The data described here indicate that VEGF-D is a
secreted protein which, like VEGF-A, binds to and

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activates VEGFR2 and can induce vascular permeability.

Example 9   Bioactivities of Internal VEGF-D Polypeptides

     The deduced amino acid sequence for VEGF-D

5   includes a central region which is similar in sequence to
all other members of the VEGF family (approximately
residues 101 to 196 of the human VEGF-D amino acid
sequence as shown in the alignment in Figure 10).
Therefore, it was thought that the bioactive portion of

10 VEGF-D might reside in the conserved region. In order to
test this hypothesis, the biosynthesis of VEGF-D was
studied, and the conserved region of human VEGF-D was
expressed in mammalian cells, purified, and tested in
bioassays as described below.

15

Plasmid construction

     A DNA fragment encoding the portion of human VEGF-
D from residue 93 to 201, *ie.* with N- and C-terminal
regions removed, was amplified by polymerase chain

20 reaction with *Pfu* DNA polymerase, using as template a
plasmid comprising full-length human VEGF-D cDNA. The
amplified DNA fragment, the sequence of which was
confirmed by nucleotide sequencing, was then inserted
into the expression vector pEFBOSSFLAG to give rise to a

25 plasmid designated pEFBOSVEGFDΔNΔC. The pEFBOSSFLAG
vector contains DNA encoding the signal sequence for
protein secretion from the interleukin-3 (IL-3) gene and
the FLAG™ octapeptide. The FLAG™ octapeptide can be
recognized by commercially available antibodies such as

30 the M2 monoclonal antibody (IBI/Kodak). The VEGF-D PCR
fragment was inserted into the vector such that the IL-3
signal sequence was immediately upstream from the FLAG™
sequence, which was in turn immediately upstream from the
VEGF-D sequence. All three sequences were in the same

35 reading frame, so that translation of mRNA resulting from
transfection of pEFBOSVEGFDΔNΔC into mammalian cells would

give rise to a protein which would have the IL-3 signal
sequence at its N-terminus, followed by the FLAG™
octapeptide and the VEGF-D sequence. Cleavage of the
signal sequence and subsequent secretion of the protein

5   from the cell would give rise to a VEGF-D polypeptide
which is tagged with the FLAG™ octapeptide adjacent to the
N-terminus. This protein was designated VEGFDΔNΔC.
        In addition, a second plasmid was constructed,
designated pEFBOSVEGFD*fullFLAG*, in which the full-length

10   coding sequence of human VEGF-D was inserted into
pEFBOSIFLAG such that the sequence for the FLAG™
octapeptide was immediately downstream from, and in the
same reading frame as, the coding sequence of VEGF-D. The
plasmid pEFBOSIFLAG lacks the IL-3 signal sequence, so

15   secretion of the VEGF-D/FLAG fusion protein was driven by
the signal sequence of VEGF-D. pEFBOSVEGFD*fullFLAG* was
designed to drive expression in mammalian cells of full-
length VEGF-D which was C-terminally tagged with the FLAG™
octapeptide. This protein is designated VEGFD*fullFLAG*, and

20   is useful for the study of VEGF-D biosynthesis.

Analysis of the Post-Translational Processing of VEGF-D
        To examine whether the VEGF-D polypeptide is
processed to give a mature and fully active protein,

25   pEFBOSVEGFD*fullFLAG* was transiently transfected into COS
cells (Aruffo and Seed, 1987). Expression in COS cells
followed by biosynthetic labeling with ^{35}S-
methionine/cysteine and immunoprecipitation with M2 gel
has demonstrated species of approximately 43 kD (*f*A) and

30   25 kD(*f*B) (Figure 12A). These bands are consistent with
the notion that VEGF-D is cleaved to give a C-terminal
fragment (FLAG™ tagged) and an internal peptide
(corresponding approximately to the VEGFDΔNΔC protein).
Reduction of the immunoprecipitates (M2*) gives some

35   reduction of the *f*A band, indicating the potential for
disulphide linkage between the two fragments.

Expression and purification of internal VEGF-D polypeptide

Plasmid pEFBOSVEGFDΔNΔC was used to transiently transfect COS cells by the DEAE-Dextran method as described previously (Aruffo and Seed, 1987). The
5   resulting conditioned cell culture medium (approximately 150 ml), collected after 7 days of incubation, was subjected to affinity chromatography using a resin to which the M2 monoclonal antibody had been coupled. In brief, the medium was run batch-wise over a 1 ml M2
10   antibody column for approximately 4 hours at 4°C. The column was then washed extensively with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl before elution with free FLAG™ peptide at 25 µg/ml in the same buffer. The resulting material was used for the bioassays described below.
15   In order to detect the purified VEGFDΔNΔC, fractions eluted from the M2 affinity column were subjected to Western blot analysis. Aliquots of the column fractions were combined with 2 x SDS-PAGE sample buffer, boiled and loaded onto a 15% SDS polyacrylamide
20   gel. The resolved fractions were transferred to nitrocellulose membrane and non-specific binding sites blocked by incubation in Tris/NaCl/Tween 20 (TST) and 10% skim milk powder (BLOTTO). Membranes were then incubated with monoclonal antibody M2 or control antibody at 3 µg/ml
25   for 2 h at room temperature, followed by extensive washing in TST. Membranes were then incubated with a secondary goat anti-mouse HRP-conjugated antiserum for 1 h at room temperature, followed by washing in TST buffer. Detection of the protein species was achieved using a
30   chemiluminescent reagent (ECL, Amersham) (Figure 12B).
Under non-reducing conditions a species of molecular weight approximately 23 kD (VEGFDΔNΔC) was detected by the M2 antibody. This is consistent with the predicted molecular weight for this internal fragment
35   (12,800) plus N-linked glycosylation; VEGFDΔNΔC contains two potential N-linked glycosylation sites. A species of

approximately 40 kD was also detected, and may represent a
non-covalent dimer of the 23 kD protein (VEGFDΔNΔC).

Bioassays

5   The bioassay for the capacity of polypeptides to
bind to VEGF receptor-2 is described in detail in
Example 7. Aliquots of fractions eluted from the M2
affinity column, containing the VEGFDΔNΔC protein, were
diluted in medium and tested in the VEGFR2 bioassay as

10   previously described. Fraction #3 from the affinity
column, which was shown to contain the purified VEGFDΔNΔC
protein (Figure 12B), demonstrated a clear ability to
induce proliferation of the bioassay cell line to a
dilution of 1/100 of the purified fraction (Figure 13).

15   In comparison, the void volume of the affinity column
(fraction #1) showed no activity, whereas the original
VEGFDΔNΔC conditioned medium gave only weak activity.

        The vascular permeability assay (Miles and Miles,
1952) is described in brief in example 8. Aliquots of

20   purified VEGFDΔNΔC, and samples of the void volume from
the M2 affinity column (negative control) were combined
with medium and injected intradermally into the skin of
guinea pigs. The regions of skin at the sites of
injections were excised, and extravasated dye was eluted.

25   The absorbance of the extravasated dye at 620 nm arising
from injection of purified VEGFDΔNΔC was 0.131 ± 0.009.

In comparison, the value for absorbance arising from
injection of a sample of the void volume was 0.092 ±
0.020. Therefore VEGFDΔNΔC induced vascular permeability,

30   but the effect was only marginal.

        Due to its ability to bind to VEGFR2 and its lower
induction of vascular permeability compared to full length
VEGF-D, VEGF–DΔNΔC may be said to relatively decrease the
induction of vascular permeability by VEGF–D through

35   competitive inhibition. In this sense, the VEGF-DΔNΔC
fragment may be thought of as an antagonist for VEGF-D as

regards the induction of vascular permeability.

Summary
        Two factors have led us to explore internal
5   fragments of VEGF-D for enhanced activity. Firstly, it is
the central region of VEGF-D which exhibits amino acid
homology with all other members of the VEGF family.
Secondly, proteolytic processing which gives rise to
internal bioactive polypeptides occurs for other growth
10   factors such as PDGF-BB. In addition, the activity seen
with the full length VEGF-D protein in COS cells was lower
than for the corresponding conditioned medium from VEGF-A
transfected COS cells.
        It was predicted that the mature VEGF-D sequence
15   would be derived from a fragment contained within residues
92-205, with cleavage at FAA^TFY and IIRR^SIQI.
Immunoprecipitation analysis of VEGF-D*full*FLAG expressed
in COS cells produced species consistent with the internal
proteolytic cleavage of the VEGF-D polypeptide at these
20   sites. Therefore a truncated form of VEGF-D, with the N-
and C-terminal regions removed (VEGFDΔNΔC), was produced
and expressed in COS cells. This protein was identified
and purified using the M2 antibody. The VEGFDΔNΔC protein
was also detected by the A2 antibody, which recognizes a
25   peptide within the 92-205 fragment of VEGF-D (not shown).
VEGFDΔNΔC was evaluated by the VEGFR2 bioassay and the
Miles vascular permeability assay, and shown to bind to
and activate the VEGFR2 receptor in a bioassay designed to
detect cross-linking of the VEGFR2 extracellular domain.
30   Induction of vascular permeability by this polypeptide in
a Miles assay was at best marginal, in contrast to the
effect of VEGF-A.

Example 10   VEGF-D Binds to and Activates VEGFR-3
35   The human VEGF-D cDNA was cloned into baculovirus
shuttle vectors for the production of reconmbinant VEGF-D.

In addition to baculoviral shuttle vectors, which
contained the unmodified VEGF-D cDNA (referred to as "full
length VEGF-D") two baculoviral shuttle vectors were
assembled, in which the VEGF-D cDNA was modified in the
5   following ways.

In one construct (referred to as "full length
VEGF-D-H_6") a C-terminal histidine tag was added. In the
other construct the putative N- and C-terminal propeptides
were removed, the melittin signal peptide was fused in-
10   frame to the N-terminus, and a histidine tag was added to
the C-terminus of the remaining VEGF homology domain
(referred to as "ΔNΔc-MELsp-VEGF-D-H_6").

For each of the three constructs baculoviral
clones of two or three independent transfections were
15   amplified . The supernatant of High Five (HF) cells was
harvested 48 h post infection with high titre virus
stocks. The supernatant was adjusted to pH 7 with NaOH
and diluted with one volume of D-MEM (0.2% FCS).

The samples were tested for their ability to
20   stimulate tyrosine phosphorylation of VEGFR-3 (Flt4
receptor) on NIH3T3 cells, as described by Joukov et al,
1996. The supernatant of uninfected cells and the
supernatant of cells infected with the short splice
variant of VEGF-C, which does not stimulate tyrosine
25   phosphorylation of VEGFR-3, were used as negative
controls. VEGF-C modified in the same way as ΔNΔC-melSP-
VEGF-D-H_6 was used as positive control. The results are
shown in Figure 14.

The appearance of new bands at 125 and 195 kD
30   indicates phosphorylation, and hence activation, of the
receptor.

Example 11 VEGF-D Binds to and Activates VEGFR-2
Modified and unmodified human VEGF-D cDNA was
35   cloned into baculovirus shuttle vectors for the production
of recombinant VEGF-D as described in Example 10.

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For each of the three constructs full length
VEGF-D, full length VEGF-D-H$_6$, and ΔNΔC-melSP-VEGF-D-H$_6$,
baculoviral clones of two or three independent
transfections were amplified . The supernatant of High

5   Five (HF) cells was harvested 48 hours post infection with
high titre virus stocks. The supernatant was adjusted to
pH 7 with NaOH and diluted with one volume of D-MEM (0.2%
FCS).

        The supernatants conditioned with the histidine-

10   tagged proteins were tested for their ability to stimulate
tyrosine phosphorylation of the KDR receptor according to
Joukov et al, 1996. KDR is the human homologue of flk1
(VEGFR-2).

        The supernatant of uninfected cells and the

15   supernatant of cells infected with the VEGF-C 156S mutant,
which does not stimulate KDR, were used as negative
controls. VEGF$_{165}$ and VEGF-C modified in the same way as
ΔNΔC-melSP-VEGF-D-H$_6$ were used as positive controls. The
results are shown in Figure 15.

20   The appearance of a new band at approximately 210
kD indicates phosphorylation, and hence activation, of the
receptor.

Example 12   Analysis of VEGF-D Gene Expression

25   In order to characterise the pattern of VEGF-D
gene expression in the human and in mouse embryos, VEGF-D
cDNAs were used as hybridization probes for Northern blot
analysis of polyadenylated human RNA and for in situ
hybridization analysis with mouse embryos.

30

Gene expression in the adult human

        A 1.1 kb fragment of the human VEGF-D cDNA shown
in Figure 4 (SEQ ID NO. 4) spanning from the *EcoRV* site to
the 3'-terminus (nucleotides 911 to 2029) was labelled

35   with [α-^{32}P]dATP using the Megaprime DNA labelling system
(Amersham) according to manufacturer's instructions. This

probe was used to screen human multiple tissue northern
blots (Clontech) by hybridization, also according to
manufacturer's instructions. These blots contained
polyadenylated RNA obtained from tissues of adult humans
5   who were apparently free of disease. Autoradiography with
the labelled blots revealed that VEGF-D mRNA was most
abundant in heart, lung and skeletal muscle. VEGF-D mRNA
was of intermediate abundance in spleen, ovary, small
intestine and colon, and was of low abundance in kidney,
10   pancreas, thymus, prostate and testis. No VEGF-D mRNA was
detected in RNA from brain, placenta, liver or peripheral
blood leukocytes. In most of the tissues where VEGF-D
mRNA was detected the size of the transcript was 2.3 kb.
The only exception was skeletal muscle, where two VEGF-D
15   transcripts of 2.3 kb and 2.8 kb were detected. In
skeletal muscle the 2.3 kb transcript was more abundant
than the 2.8 kb transcript.

Gene expression in mouse embryos
20   In order to generate an antisense RNA probe for
mouse VEGF-D mRNA, the mouse VEGF-D2 cDNA shown in Figure
7 (SEQ ID NO. 7) was inserted into the transcription
vector pBluescriptIIKS+ (Stratagene). The resulting
plasmid was digested to completion with the restriction
25   endonuclease *Fok*I and then used as template for an *in
vitro* transcription reaction with T3 RNA polymerase. This
transcription reaction gave rise to an antisense RNA probe
for VEGF-D mRNA which was complementary in sequence to the
region of the VEGF-D2 cDNA (Figure 7) from the 3'-terminus
30   to the *Fok*I cleavage site closest to the 3'-terminus
(nucleotides 1135 to 700). This antisense RNA probe was
hybridized at high stringency with paraffin-embedded
tissue sections generated from mouse embryos at post-
coital day 15.5. Hybridization and washing were
35   essentially as described previously (Achen *et al.*, 1995).
        After washing and drying, slides were exposed to
autoradiography film for six days.

Development of the autoradiography film revealed
that VEGF-D mRNA is localised in the developing lung of
post-coital day 15.5 embryos. The signal for VEGF-D mRNA
in the lung was strong and highly specific. Control

5   hybridizations with sense probe gave no detectable
background in lung or any other tissue.

Summary

The VEGF-D gene is broadly expressed in the adult

10   human, but is certainly not ubiquitously expressed.
Strongest expression was detected in heart, lung and
skeletal muscle. In mouse embryos at post-coital day
15.5, strong and specific expression of the VEGF-D gene
was detected in the lung. These data suggest that VEGF-D

15   may play a role in lung development, and that expression
of the VEGF-D gene in lung persists in the adult, at least
in humans. Expression of the gene in other tissues in the
adult human suggests that VEGF-D may fulfill other
functions in other adult tissues.

20

Example 13   VEGF-D is Mitogenic for Endothelial Cells

Some members of the VEGF family of proteins,
namely VEGF-A (Leung *et al*, 1989) and VEGF-B (Olofsson *et
al*, 1996), are mitogenic for endothelial cells. In order

25   to test the mitogenic capacity of VEGFDΔNΔC for
endothelial cells, this protein was expressed and purified
by affinity chromatography as described in Example 9.
Fraction #3, eluted from the M2 affinity column, which
contained VEGFDΔNΔC, was diluted 1 in 10 in cell culture

30   medium containing 5% serum and applied to bovine aortic
endothelial cells (BAEs) which had been propagated in
medium containing 10% serum. The BAEs had been seeded in
24-well dishes at a density of 10,000 cells per well the
day before addition of VEGFDΔNΔC, and 3 days after

35   addition of this polypeptide the cells were dissociated
with trypsin and counted. Purified VEGF-A was included in

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the experiment as positive control. Results are shown in
Figure 16. The addition of fraction #3 to the cell
culture medium led to a 2.4-fold increase in the number of
BAEs after 3 days of incubation, a result which was

5   comparable to that obtained with VEGF-A. Clearly
VEGFDΔNΔC is mitogenic for endothelial cells.

Example 14 Localization of the VEGF-D Gene on Human
           Chromosomes

10   In order to generate hybridization probes for
localization of the VEGF-D gene on human chromosomes, a
human genomic DNA clone for VEGF-D was isolated from a
human genomic DNA library (Clontech). The genomic library
was screened by hybridization with the human VEGF-D cDNA

15   shown in Figure 4, using standard methods (Sambrook et
al., 1989). One of the clones thus isolated was shown to
contain part of the VEGF-D gene by hybridization to
numerous oligonucleotides which were derived in sequence
from the human VEGF-D cDNA. A region of the genomic

20   clone, approximately 13 kb in size, was purified from
agarose gel, labelled by nick-translation with biotin-14-
dATP and hybridized *in situ* at a final concentration of 20
ng/μl to metaphases from two normal human males. The
fluorescence *in situ* hybridization (FISH) method was

25   modified from that previously described (Callen *et al*,
1990) in that chromosomes were stained before analysis
with propidium iodide (as counterstain) and DAPI (for
chromosome identification). Images of metaphase
preparations were captured by a cooled CCD camera, using

30   the CytoVision Ultra image collection and enhancement
system (Applied Imaging Int. Ltd.). FISH signals and the
DAPI banding pattern were merged for analysis.
            Fifteen metaphases from the first normal male were
examined for fluorescent signal. Ten of the metaphases

35   showed signal on one chromatid (3 cells) or both
chromatids (7 cells) of the X chromosome in band p22.1.

There was a total of 9 non-specific background dots
observed in these 15 metaphases. A similar result was
obtained from hybridization of the probe to 15 metaphases
from the second normal male, where signal was observed at
5   Xp22.1 on one chromatid in 7 cells and on both chromatids
in 4 cells. In conclusion, the human VEGF-D gene is
located on the X chromosome in band p22.1.

Example 15   Localization of the murine VEGF-D Gene on
10   Mouse Chromosomes
        The mouse chromosomal location of the VEGF-D gene
was determined by interspecific backcross analysis using
progeny generated by mating (C57BL/6J x Mus spretus)F1
females and CB7BL/67 males as described previously
15   (Copeland and Jenkins, 1991). This interspecific
backcross mapping panel has been typed for over 2400 loci
that are well distributed among all the autosomes as well
as the X chromosome (Copeland and Jenkins, 1991).
C57BL/6J and M. spretus DNAs were digested with several
20   enzymes and analyzed by Southern blot hybridization for
informative restriction fragment length polymorphisms
(RFLPs) using a 1.3 kb mouse VEGF-D cDNA probe essentially
as described (Jenkins et al. 1982). Fragments of 7.1,
6.3, 4.7, 2.5 and 2.2 kb were detected in TaqI-digested
25   C57BL/6J DNA and major fragments of 7.1, 3.7, 2.7 and 2.2
kb were detected in TaqI-digested M. spretus DNA. The
presence or absence of the 3.7 and 2.7 TaqI M. spretus-
specific fragments, which cosegregated, was followed in
backcross mice. The mapping results indicated that the
30   VEGF-D gene is located in the distal region of the mouse X
chromosome linked to Bik, DxPasI and Ptmb4. Although 89
mice were analyzed for every marker, up to 133 mice were
typed for some pairs of markers. Each locus was analyzed
in pairwise combinations for recombination frequencies
35   using the additional data. The ratios of the total number
of mice exhibiting recombinant chromosomes to the total

number of mice analyzed for each pair of loci and the most
likely gene order are: centromere - *Btk* - 14/121 - *DxPasI*
- 3/99 - VEGF-D - 5/133 - *Ptmb4*. The recombination
frequencies [expressed as genetic distances in
5   centiMorgans (cM) ± the standard error], calculated using
Map Manager (version 2.6.5), are - *Btk* - 11.6+/—2.9 -
DxPasI - 3.0+/-1.7 - VEGF-D - 3.8+/-1.7 - *Ptmb4*. A
description of the probes and RFLPs for the loci linked to
the VEGF-D gene, including *Btk*, *DxPasI* and *Ptmb4*, has been
10   reported previously (Hacfliger *et al.*, 1992; Holloway *et
al.*, 1997).

          We have compared our interspecific map of the X
chromosome with a composite mouse linkage map that reports
the map location of many uncloned mutations (provided from
15   Mouse Genome Database, a computerized database maintained
at The Jackson Library, Bar Harbor, ME). The VEGF-D gene
mapped in a region of the composite map that lacks mouse
mutations with a phenotype that might be expected for an
alteration in the locus for an endothelial cell mitogen.
20   The distal region of the mouse X-chromosome shares a
region of homology with the short arm of the human X
chromosomes (Mouse Genome Database). The placement of the
VEGF-D gene in this interval in mouse suggests that the
human homolog will map to Xp22. This is consistent with
25   our FISH analysis which has localized the human gene to
Xp22.1.

          Numerous disease states are caused by mutations in
unknown genes which have been mapped to Xp22.1 and the
positions immediately surrounding this region in the
30   human. These disease states include Kallmann syndrome,
ocular albinism (Nettleship-Falls type), ocular albinism
and sensorineural deafness, Partington syndrome,
spondyloepiphyseal dysplasia (late), retinitis pigmentosa
15, gonadal dysgenesis (XY female type), Nance-Horan
35   cataract-dental syndrome, retinoschisis, Charcot-Marie-
Tooth disease, F-cell production, hypomagnesemia,
keratosis follicularis spinulosa decalvans, Coffin-Lowry

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syndrome, corneal dermoids, hypophosphatemia, agammaglobulinemia, Aicardi symdrome, hereditary hypophosphatemia II, mental retardation (non-dysmorphic), Opitz G syndrome, pigment disorder (reticulate), dosage-
5   sensitive sex reversal, adrenal hypoplasia, retinitis pigmentosa-6, deafness 4 (congenital sensorineural) and Wilson-Turner syndrome. The positions of the genes involved in these disease states are documented in the OMIM gene map which is edited by Dr. Victor McKusick and
10   colleagues at Johns Hopkins University (USA).

BIOASSAYS TO DETERMINE THE FUNCTION OF VEGF-D

        Other assays are conducted to evaluate whether VEGF-D has similar activities to VEGF in relation to
15   endothelial cell function, angiogenesis and wound healing. Further assays may also be performed, depending on the results of receptor binding distribution studies.

I.   **Assays of Endothelial Cell Function**
20   a)   *Endothelial cell proliferation*
        Endothelial cell growth assays are performed by methods well known in the art, *eg.* those of Ferrara & Henzel (1989), Gospodarowicz *et al* (1989), and/or Claffey *et al*, Biochim. Biophys. Acta, 1995 1246 1-9.
25

b)   *Cell adhesion assay*
        The effect of VEGF-D on adhesion of polmorphonuclear granulocytes to endothelial cells is tested.
30

c)   *Chemotaxis*
        The standard Boyden chamber chemotaxis assay is used to test the effect of VEGF-D on chemotaxis.

35   d)   *Plasminogen activator assay*
        Endothelial cells are tested for the effect of VEGF-D on plasminogen activator and plasminogen activator

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inhibitor production, using the method of Pepper *et al* (1991).

e)   *Endothelial cell Migration assay*

The ability of VEGF-D to stimulate endothelial cells to migrate and form tubes is assayed as described in Montesano *et al* (1986). Alternatively, the three-dimensional collagen gel assay described by Joukov *et al* (1996) or a gelatinized membrane in a modified Boyden chamber (Glaser *et al*, 1980) may be used.

II   Angiogenesis Assay

The ability of VEGF-D to induce an angiogenic response in chick chorioallantoic membrane is tested as described in Leung *et al* (1989). Alternatively the rat cornea assay of Rastinejad *et al* (1989) may be used; this is an accepted method for assay of *in vivo* angiogenesis, and the results are readily transferrable to other *in vivo* systems.

III   Wound Healing

The ability of VEGF-D to stimulate wound healing is tested in the most clinically relevant model available, as described in Schilling *et al* (1959) and utilised by Hunt *et al* (1967).

IV   The Haemopoietic System

A variety of *in vitro* and *in vivo* assays using specific cell populations of the haemopoietic system are known in the art, and are outlined below. In particular a variety of *in vitro* murine stem cell assays using fluorescence-activated cell sorter purified cells are particularly convenient:

a)   *Repopulating Stem Cells*

These are cells capable of repopulating the bone marrow of lethally irradiated mice, and have the Lin⁻,

Rh^{h1}, Ly-6A/E$^+$, c-kit$^+$ phenotype. VEGF-D is tested on these cells either alone, or by co-incubation with other factors, followed by measurement of cellular proliferation by ^{3}H-thymidine incorporation.

5

b)   *Late Stage Stem Cells*

These are cells that have comparatively little bone marrow repopulating ability, but can generate D13 CFU-S. These cells have the Lin$^-$, Rh^{hl}, Ly-6A/E$^+$, c-kit$^+$

10   phenotype. VEGF-D is incubated with these cells for a period of time, injected into lethally irradiated recipients, and the number of D13 spleen colonies enumerated.

15   c)   *Progenitor-Enriched Cells*

These are cells that respond *in vitro* to single growth factors and have the Lin$^-$, Rh^{hl}, Ly-6A/E$^+$, c-kit$^+$ phenotype. This assay will show if VEGF-D can act directly on haemopoietic progenitor cells. VEGF-D is

20   incubated with these cells in agar cultures, and the number of colonies present after 7-14 days is counted.

V   **Atherosclerosis**

Smooth muscle cells play a crucial role in the

25   development or initiation of atherosclerosis, requiring a change of their phenotype from a contractile to a synthetic state. Macrophages, endothelial cells, T lymphocytes and platelets all play a role in the development of atherosclerotic plaques by influencing the

30   growth and phenotypic modulations of smooth muscle cell. An *in vitro* assay using a modified Rose chamber in which different cell types are seeded on to opposite coverslips measures the proliferative rate and phenotypic modulations of smooth muscle cells in a multicellular environment, and

35   is used to assess the effect of VEGF-D on smooth muscle cells.

VI   **Metastasis**

The ability of VEGF-D to inhibit metastasis is
assayed using the Lewis lung carcinoma model, for example
using the method of Cao *et al* (1995).

5

VII   **VEGF-D in Other Cell Types**

The effects of VEGF-D on proliferation,
differentiation and function of other cell types, such as
liver cells, cardiac muscle and other cells, endocrine
10   cells and osteoblasts can readily be assayed by methods
known in the art, such as ^{3}H-thymidine uptake by *in vitro*
cultures. Expression of VEGF-D in these and other tissues
can be measured by techniques such as Northern blotting
and hybridization or by *in situ* hybridization.

15

VIII   **Construction of VEGF-D Variants and Analogues**

VEGF-D is a member of the PDGF family of growth
factors which exhibits a high degree of homology to the
other members of the PDGF family. VEGF-D contains eight
20   conserved cysteine residues which are characteristic of
this family of growth factors. These conserved cysteine
residues form intra-chain disulfide bonds which produce
the cysteine knot structure, and inter-chain disulfide
bonds that form the protein dimers which are
25   characteristic of members of the PDGF family of growth
factors. VEGF-D will interact with protein tyrosine
kinase growth factor receptors.

In contrast to proteins where little or nothing is
known about the protein structure and active sites needed
30   for receptor binding and consequent activity, the design
of active mutants of VEGF-D is greatly facilitated by the
fact that a great deal is known about the active sites and
important amino acids of the members of the PDGF family of
growth factors.

35   Published articles elucidating the
structure/activity relationships of members of the PDGF
family of growth factors include for PDGF: Oestman *et al*,

J. Biol. Chem., 1991 266 10073-10077; Andersson *et al*, J.
Biol. Chem., 1992 267 11260-1266; Oefner *et al*, EMBO J.,
1992 11 3921-3926; Flemming *et al*, Molecular and Cell
Biol., 1993 13 4066-4076 and Andersson *et al*, Growth
5   Factors, 1995 12 159-164; and for VEGF: Kim *et al*, Growth
Factors, 1992 7 53-64; Pötgens *et al*, J. Biol. Chem., 1994
269 32879-32885 and Claffey *et al*, Biochem. Biophys. Acta,
1995 1246 1-9. From these publications it is apparent
that because of the eight conserved cysteine residues, the
10   members of the PDGF family of growth factors exhibit a
characteristic knotted folding structure and dimerization,
which result in formation of three exposed loop regions at
each end of the dimerized molecule, at which the active
receptor binding sites can be expected to be located.

15   Based on this information, a person skilled in the
biotechnology arts can design VEGF-D mutants with a very
high probability of retaining VEGF-D activity by
conserving the eight cysteine residues responsible for the
knotted folding arrangement and for dimerization, and also
20   by conserving, or making only conservative amino acid
substitutions in the likely receptor sequences in the
loop 1, loop 2 and loop 3 region of the protein structure.

                The formation of desired mutations at specifically
targeted sites in a protein structure is considered to be
25   a standard technique in the arsenal of the protein chemist
(Kunkel *et al*, Methods in Enzymol., 1987 154 367-382).
Examples of such site-directed mutagenesis with VEGF can
be found in Pötgens *et al*, J. Biol. Chem., 1994 269 32879-
32885 and Claffey *et al*, Biochim. Biophys. Acta, 1995 1246
30   1-9. Indeed, site-directed mutagenesis is so common that
kits are commercially available to facilitate such
procedures (*eg*. Promega 1994-1995 Catalog., Pages 142-
145).

                The endothelial cell proliferating activity of
35   VEGF-D mutants can be readily confirmed by well
established screening procedures. For example, a

procedure analogous to the endothelial cell mitotic assay
described by Claffey *et al*, (Biochim. Biophys. Acta., 1995
__1246__ 1-9) can be used. Similarly the effects of VEGF-D on
proliferation of other cell types, on cellular

5   differentiation and on human metastasis can be tested
using methods which are well known in the art.

It will be apparent to the person skilled in the
art that while the invention has been described in some
detail for the purposes of clarity and understanding,

10   various modifications and alterations to the embodiments
and methods described herein may be made without departing
from the scope of the inventive concept disclosed in this
specification.

References cited herein are listed on the

15   following pages, and are incorporated herein by this
reference.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: LUDWIG INSTITUTE FOR CANCER RESEARCH

    (ii) TITLE OF INVENTION: GROWTH FACTOR

    (iii) NUMBER OF SEQUENCES: 11

    (iv) CORRESPONDENCE ADDRESS:
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        (C) CITY: Washington
        (D) STATE: DC
        (E) COUNTRY: United States of America
        (F) ZIP: 20005

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: EVANS, Joseph D.
        (C) REFERENCE/DOCKET NUMBER: 1064/42983PCT

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (202) 628-8800
        (B) TELEFAX: (202) 628-8844
        (C) TELEX: N/A

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 2846 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (vi) ORIGINAL SOURCE:
        (F) TISSUE TYPE: Human Breast

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GGAATTCAGT GAAGTAAGAA AGACAAAGTG TTCATTGGAG ATTTTTAGTA AGGGGCCAAC      60

AGAGCTGCTA AAGTCATGCT TCACTTAACG ATGGGGATAT GTTCGGAGAA ATGCATTGTT     120

AGGTGATTTT GTCGTTGTGC AAGCATCTTA GAGTACACTT AGACAAACCT AGCTGGTATA     180

ACCTAGGTGT GTAGTAGGAT ATATGGTATA GCCTATTGTT CCTAGGCTAC AAACCCATAC     240

AGCATGTTCC TGTACTGAAT ACTGAGGCAA CTGCAACACC GTGGTGAGTA TTTGTGTATC     300

TAAACATACC TAAACATAGA AAAGATACAG TAAAAATATG GCATTATAGT CTTATGGGAC     360
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TACTGTCATA CATACAGTCC ATATATTGTT GACTGTGTAA TGTTGACCTG AATGTCATTA    420

TGTGGCAGGC ACATGACTGT GTCGCTAACC TTTGCACAAG ATTACTGTAG GATTACATGA    480

GATAGTTGTA AATAATTGGT GGGGTACTGG GCACCTAGTA GGTATGCATA CATGTTCACC    540

ATCATTATGG TTGTTTTAAA TCACCTAACC CAGGCCCTGC ACATAGTAAG ACATCAACAA    600

ATTGTAGCTG CTACTATTTT GCGCATCTAA TCTTAATATC ATTTATTTTG TAGTCCTTGG    660

ATGTTCCCTC CTTTATGACT TCTTTTTTTT TTGTTGTCCT TCCTTTAGCC CTCCATCCTC    720

TACAGCTCAG CATCAGAACA CTCTCTTTTT AGACTCCGAT ATGGGGTCCT CCAAGAAAGT    780

TACTCTCTCA GTGCTCAGCC GGGAGCAGTC GGAAGGGGTT GGAGCGAGGG TCCGGAGAAG    840

CATTGGCAGA CCCGAGTTAA AAAATCTGGA TCCGTTTTTA CTGTTTGATG AATTTAAAGG    900

AGGTAGACCA GGAGGATTTC CTGATCATCC ACATCGAGGT TTTGAAACAG TATCCTACCT    960

CCTGGAAGGG GGCAGCATGG CCCATGAAGA CTTCTGTGGA CACACTGGTA AAATGAACCC   1020

AGGAGATTTG CAGTGGATGA CTGCGGGCCG GGGCATTCTG CACGCTGAGA TGCCTTGCTC   1080

AGAGGAGCCA GCCCATGGCC TACAACTGTG GGTTAATTTG AGGAGCTCAG AGAAGATGGT   1140

GGAGCCTCAG TACCAGGAAC TGAAAAGTGA AGAAATCCCT AAACCCAGTA AGGATGGTGT   1200

GACAGTTGCT GTCATTTCTG GAGAAGCCCT GGGAATAAAG TCCAAGGTTT ACACTCGCAC   1260

ACCAACCTTA TATTTGGACT TCAAATTGGA CCCAGGAGCC AAACATTCCC AACCTATCCC   1320

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ATCCCAGCAC AGGTATGCCC AGGGCAGGGT GCCTTTCAGC TTACAGAACA TTCAGTGAGG   1440

GAAGAGAATA TGAACACCAG TCATGACACA TCCTGTGCAC AGATGAAAGT CCAGGCACCA   1500

TTATGTGTTT TGATACCTCG CTAAGACGTT GGCAACCTCC ATACTGATAA AGGGATGGAG   1560

CTACAGTGGA CTCCAAGGGG AGCAGGAATC TGCCTATCTC CTGGGAGAAG GAAATGGAAG   1620

GAGGGCCCGA TGATGCACAA CAAAAAATAG AACCTCATCA CACAGCAGTG CTTGGAGAAG   1680

GTGACAGTGT CCAAGTGGAG AACAAGGATC CCAAGAGAAG CCACTTTGTC TTAATTGCTG   1740

GGGAGCCATT AAGAGAACCA GTTATCCAAC ATGCGATCAT CTCAGTCCAC ATTGGAACGA   1800

TCTGAACAGC AGATCAGGGC TGCTTCTAGT TTGGAGGAAC TACTTCGAAT TACTCACTCT   1860

GAGGACTGGA AGCTGTGGAG ATGCAGGCTG AGGCTCAAAA GTTTTACCAG TATGGACTCT   1920

CGCTCAGCAT CCCATCGGTC CACTAGGTTT GCGGCAACTT TCTATGACAT TGAAACACTA   1980

AAAGTTATAG ATGAAGAATG GCAAAGAACT CAGTGCAGCC CTAGAGAAAC GTGCGTGGAG   2040

GTGGCCAGTG AGCTGGGGAA GAGTACCAAC ACATTCTTCA AGCCCCCTTG TGTGAACGTG   2100

TTCCGATGTG GTGGCTGTTG CAATGAAGAG AGCCTTATCT GTATGAACAC CAGCACCTCG   2160

TACATTTCCA AACAGCTCTT TGAGATATCA GTGCCTTTGA CATCAGTACC TGAATTAGTG   2220

CCTGTTAAAG TTGCCAATCA TACAGGTTGT AAGTGCTTGC CAACAGCCCC CCGCCATCCA   2280

TACTCAATTA TCAGAAGATC CATCCAGATC CCTGAAGAAG ATCGCTGTTC CCATTCCAAG   2340

AAACTCTGTC CTATTGACAT GCTATGGGAT AGCAACAAAT GTAAATGTGT TTTGCAGGAG   2400
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GAAAATCCAC TCGCTGGAAC AGAAGACCAC TCTCATCTCC AGGAACCAGC TCTCTGTGGG   2460

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GATCTAATCC AGCACCCCAA AAACTGCAGT TGCTTTGAGT GCAAAGAAAG TCTGGAGACC   2580

TGCTGCCAGA AGCACAAGCT ATTTCACCCA GACACCTGCA GCTGTGAGGA CAGATGCCCC   2640

TTTCATACCA GACCATGTGC AAGTGGCAAA ACAGCATGTG CAAAGCATTG CCGCTTTCCA   2700

AAGGAGAAAA GGGCTGCCCA GGGGCCCCAC AGCCGAAAGA ATCCTTGATT CAGCGTTCCA   2760

AGTTCCCCAT CCCTGTCATT TTTAACAGCA TGCTGCTTTG CCAAGTTGCT GTCACTGTTT   2820

TTTTCCCAGG TGTTAAAAAA AAAAAA   2846

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 13 amino acids
             (B) TYPE: amino acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: peptide

      (iii) HYPOTHETICAL: NO

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

        Pro Xaa Cys Val Xaa Xaa Xaa Arg Cys Xaa Gly Cys Cys
        1   5   10

(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 325 amino acids
             (B) TYPE: amino acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: protein

      (iii) HYPOTHETICAL: NO

       (vi) ORIGINAL SOURCE:
             (F) TISSUE TYPE: Human Breast

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

        Met Arg Ser Ser Gln Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg
        1   5   10   15

        Ala Ala Ser Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp
                        20   25   30

        Trp Lys Leu Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met
                    35   40   45

        Asp Ser Arg Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe
            50   55   60

        Tyr Asp Ile Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr
        65   70   75   80

```
Gln Cys Ser Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly
             85                  90                  95

Lys Ser Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg
            100                 105                 110

Cys Gly Gly Cys Cys Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser
            115                 120                 125

Thr Ser Tyr Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr
    130                 135                 140

Ser Val Pro Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys
145                 150                 155                 160

Lys Cys Leu Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg
                165                 170                 175

Ser Ile Gln Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu
                180                 185                 190

Cys Pro Ile Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu
            195                 200                 205

Gln Glu Glu Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln
    210                 215                 220

Glu Pro Ala Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys
225                 230                 235                 240

Glu Cys Val Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro
                245                 250                 255

Lys Asn Cys Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys
                260                 265                 270

Gln Lys His Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg
        275                 280                 285

Cys Pro Phe His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala
    290                 295                 300

Lys His Cys Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His
305                 310                 315                 320

Ser Arg Lys Asn Pro
                325
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(2) INFORMATION FOR SEQ ID NO:4:

      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 2029 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

     (vi) ORIGINAL SOURCE:
            (F) TISSUE TYPE: Human Lung

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTTGGGTTCC AGCTTTCTGT AGCTGTAAGC ATTGGTGGCC ACACCACCTC CTTACAAAGC   60

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AACTAGAACC TGCGGCATAC ATTGGAGAGA TTTTTTTAAT TTTCTGGACA TGAAGTAAAT      120

TTAGAGTGCT TTCTAATTTC AGGTAGAAGA CATGTCCACC TTCTGATTAT TTTTGGAGAA      180

CATTTTGATT TTTTTCATCT CTCTCTCCCC ACCCCTAAGA TTGTGCAAAA AAAGCGTACC      240

TTGCCTAATT GAAATAATTT CATTGGATTT TGATCAGAAC TGATTATTTG GTTTTCTGTG      300

TGAAGTTTTG AGGTTTCAAA CTTTCCTTCT GGAGAATGCC TTTTGAAACA ATTTTCTCTA      360

GCTGCCTGAT GTCAACTGCT TAGTAATCAG TGGATATTGA AATATTCAAA ATGTACAGAG      420

AGTGGGTAGT GGTGAATGTT TTCATGATGT TGTACGTCCA GCTGGTGCAG GGCTCCAGTA      480

ATGAACATGG ACCAGTGAAG CGATCATCTC AGTCCACATT GGAACGATCT GAACAGCAGA      540

TCAGGGCTGC TTCTAGTTTG GAGGAACTAC TTCGAATTAC TCACTCTGAG GACTGGAAGC      600

TGTGGAGATG CAGGCTGAGG CTCAAAAGTT TTACCAGTAT GGACTCTCGC TCAGCATCCC      660

ATCGGTCCAC TAGGTTTGCG GCAACTTTCT ATGACATTGA AACACTAAAA GTTATAGATG      720

AAGAATGGCA AAGAACTCAG TGCAGCCCTA GAGAAACGTG CGTGGAGGTG GCCAGTGAGC      780

TGGGAAGAG TACCAACACA TTCTTCAAGC CCCCTTGTGT GAACGTGTTC CGATGTGGTG       840

GCTGTTGCAA TGAAGAGAGC CTTATCTGTA TGAACACCAG CACCTCGTAC ATTTCCAAAC      900

AGCTCTTTGA GATATCAGTG CCTTTGACAT CAGTACCTGA ATTAGTGCCT GTTAAAGTTG      960

CCAATCATAC AGGTTGTAAG TGCTTGCCAA CAGCCCCCCG CCATCCATAC TCAATTATCA     1020

GAAGATCCAT CCAGATCCCT GAAGAAGATC GCTGTTCCCA TTCCAAGAAA CTCTGTCCTA     1080

TTGACATGCT ATGGGATAGC AACAAATGTA AATGTGTTTT GCAGGAGGAA AATCCACTTG     1140

CTGGAACAGA AGACCACTCT CATCTCCAGG AACCAGCTCT CTGTGGGCCA CACATGATGT     1200

TTGACGAAGA TCGTTGCGAG TGTGTCTGTA AAACACCATG TCCCAAAGAT CTAATCCAGC     1260

ACCCCAAAAA CTGCAGTTGC TTTGAGTGCA AAGAAAGTCT GGAGACCTGC TGCCAGAAGC     1320

ACAAGCTATT TCACCCAGAC ACCTGCAGCT GTGAGGACAG ATGCCCCTTT CATACCAGAC     1380

CATGTGCAAG TGGCAAAACA GCATGTGCAA AGCATTGCCG CTTTCCAAAG GAGAAAAGGG     1440

CTGCCCAGGG GCCCCACAGC CGAAAGAATC CTTGATTCAG CGTTCCAAGT TCCCCATCCC     1500

TGTCATTTTT AACAGCATGC TGCTTTGCCA AGTTGCTGTC ACTGTTTTTT TCCCAGGTGT     1560

TAAAAAAAAA ATCCATTTTA CACAGCACCA CAGTGAATCC AGACCAACCT TCCATTCACA     1620

CCAGCTAAGG AGTCCCTGGT TCATTGATGG ATGTCTTCTA GCTGCAGATG CCTCTGCGCA     1680

CCAAGGAATG GAGAGGAGGG GACCCATGTA ATCCTTTTGT TTAGTTTTGT TTTTGTTTTT     1740

TGGTGAATGA GAAAGGTGTG CTGGTCATGG AATGGCAGGT GTCATATGAC TGATTACTCA     1800

GAGCAGATGA GGAAAACTGT AGTCTCTGAG TCCTTTGCTA ATCGCAACTC TTGTGAATTA     1860

TTCTGATTCT TTTTTATGCA GAATTTGATT CGTATGATCA GTACTGACTT TCTGATTACT     1920

GTCCAGCTTA TAGTCTTCCA GTTTAATGAA CTACCATCTG ATGTTTCATA TTTAAGTGTA     1980

TTTAAAGAAA ATAAACACCA TTATTCAAGC CAAAAAAAAA AAAAAAAA               2029
```

(2) INFORMATION FOR SEQ ID NO:5:

        (i) SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 354 amino acids
               (B) TYPE: amino acid
               (C) STRANDEDNESS: single
               (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: protein

      (iii) HYPOTHETICAL: NO

       (vi) ORIGINAL SOURCE:
               (F) TISSUE TYPE: Human Lung

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Tyr Arg Glu Trp Val Val Val Asn Val Phe Met Met Leu Tyr Val
1   5   10   15

Gln Leu Val Gln Gly Ser Ser Asn Glu His Gly Pro Val Lys Arg Ser
            20   25   30

Ser Gln Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser
        35   40   45

Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp Trp Lys Leu
    50   55   60

Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp Ser Arg
65   70   75   80

Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile
            85   90   95

Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser
            100   105   110

Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Ser Thr
        115   120   125

Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly
    130   135   140

Cys Cys Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser Tyr
145   150   155   160

Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro
            165   170   175

Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu
            180   185   190

Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln
        195   200   205

Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile
    210   215   220

Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Glu
225   230   235   240

Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala
            245   250   255

Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val
            260   265   270

- 60 -

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Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys
        275             280                 285
```

```
Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His
    290                 295             300
```

```
Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe
305             310             315             320
```

```
His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys
                325             330             335
```

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Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys
            340             345             350
```

```
Asn Pro
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(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 1325 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (vi) ORIGINAL SOURCE:
       (F) TISSUE TYPE: Mouse Lung

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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GGAGAATGCC TTTTGCAACA CTTTTCAGTA GCTGCCTGGA AACAACTGCT TAGTCATCGG      60

TAGACATTTA AAATATTCAA AATGTATGGA GAATGGGGAA TGGGGAATAT CCTCATGATG     120

TTCCATGTGT ACTTGGTGCA GGGCTTCAGG AGCGAACATG GACCAGTGAA GGATTTTTCT     180

TTTGAGCGAT CATCCCGGTC CATGTTGGAA CGATCTGAAC AACAGATCCG AGCAGCTTCT     240

AGTTTGGAGG AGTTGCTGCA AATCGCGCAC TCTGAGGACT GGAAGCTGTG GCGATGCCGG     300

TTGAAGCTCA AAAGTCTTGC CAGTATGGAC TCACGCTCAG CATCCCATCG CTCCACCAGA     360

TTTGCGGCAA CTTTCTATGA CACTGAAACA CTAAAAGTTA TAGATGAAGA ATGGCAGAGG     420

ACCCAATGCA GCCCTAGAGA GACATGCGTA GAAGTCGCCA GTGAGCTGGG GAAGACAACC     480

AACACATTCT TCAAGCCCCC CTGTGTAAAT GTCTTCCGGT GTGGAGGCTG CTGCAACGAA     540

GAGGGTGTGA TGTGTATGAA CACAAGCACC TCCTACATCT CCAAACAGCT CTTTGAGATA     600

TCAGTGCCTC TGACATCAGT GCCCGAGTTA GTGCCTGTTA AAATTGCCAA CCATACGGGT     660

TGTAAGTGCT TGCCCACGGG CCCCCGCCAT CCTTACTCAA TTATCAGAAG ATCCATTCAG     720

ACCCCAGAAG AAGATGAATG TCCTCATTCC AAGAAACTCT GTCCTATTGA CATGCTGTGG     780

GATAACACCA AATGTAAATG TGTTTTGCAA GACGAGACTC CACTGCCTGG GACAGAAGAC     840

CACTCTTACC TCCAGGAACC CACTCTCTGT GGACCGCACA TGACGTTTGA TGAAGATCGC     900

TGTGAGTGCG TCTGTAAAGC ACCATGTCCG GGAGATCTCA TTCAGCACCC GGAAAACTGC     960
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AGTTGCTTTG AGTGCAAAGA AAGTCTGGAG AGCTGCTGCC AAAAGCACAA GATTTTTCAC        1020

CCAGACACCT GCAGCTGTGA GGACAGATGT CCTTTTCACA CCAGAACATG TGCAAGTAGA        1080

AAGCCAGCCT GTGGAAAGCA CTGGCGCTTT CCAAAGGAGA CAAGGGCCCA GGGACTCTAC        1140

AGCCAGGAGA ACCCTTGATT CAACTTCCTT TCAAGTCCCC CCATCTCTGT CATTTTAAAC        1200

AGCTCACTGC TTTGTCAAGT TGCTGTCACT GTTGCCCACT ACCCCTTGAA CATGTGCAAA        1260

CACAGACACA CACACACACA CACACACAGA GCAACTAGAA TTATGTTTTC TAGGTGCTGC        1320

                                                                         1325
CTAAG
```

(2) INFORMATION FOR SEQ ID NO:7:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 1135 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

     (vi) ORIGINAL SOURCE:
          (F) TISSUE TYPE: Mouse Lung

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
AAACTTTGCT TCTGGAGAAT GCCTTTTGCA ACACTTTTCA GTAGCTGCCT GGAAACAACT         60

GCTTAGTCAT CGGTAGACAT TTAAAATATT CAAAATGTAT GGAGAATGGG GAATGGGGAA        120

TATCCTCATG ATGTTCCATG TGTACTTGGT GCAGGGCTTC AGGAGCGAAC ATGGACCAGT        180

GAAGCGATCA TCCCGGTCCA TGTTGGAACG ATCTGAACAA CAGATCCGAG CAGCTTCTAG        240

TTTGGAGGAG TTGCTGCAAA TCGCGCACTC TGAGGACTGG AAGCTGTGGC GATGCCGGTT        300

GAAGCTCAAA AGTCTTGCCA GTATGGACTC ACGCTCAGCA TCCCATCGCT CCACCAGATT        360

TGCGGCAACT TTCTATGACA CTGAAACACT AAAAGTTATA GATGAAGAAT GGCAGAGGAC        420

CCAATGCAGC CCTAGAGAGA CATGCGTAGA AGTCGCCAGT GAGCTGGGGA AGACAACCAA        480

CACATTCTTC AAGCCCCCCT GTGTAAATGT CTTCCGGTGT GGAGGCTGCT GCAACGAAGA        540

GGGTGTGATG TGTATGAACA CAAGCACCTC CTACATCTCC AAACAGCTCT TTGAGATATC        600

AGTGCCTCTG ACATCAGTGC CCGAGTTAGT GCCTGTTAAA ATTGCCAACC ATACGGGTTG        660

TAAGTGCTTG CCCACGGGCC CCCGCCATCC TTACTCAATT ATCAGAAGAT CCATTCAGAC        720

CCCAGAAGAA GATGAATGTC CTCATTCCAA GAAACTCTGT CCTATTGACA TGCTGTGGGA        780

TAACACCAAA TGTAAATGTG TTTTGCAAGA CGAGACTCCA CTGCCTGGGA CAGAAGACCA        840

CTCTTACCTC CAGGAACCCA CTCTCTGTGG ACCGCACATG ACGTTTGATG AAGATCGCTG        900

TGAGTGCGTC TGTAAAGCAC CATGTCCGGG AGATCTCATT CAGCACCCGG AAAACTGCAG        960

TTGCTTTGAG TGCAAAGAAA GTCTGGAGAG CTGCTGCCAA AAGCACAAGA TTTTTCACCC       1020

AGACACCTGC AGGTCAATGG TCTTTTCGCT TTCCCCTTAA CTTGGTTTAC TGATGACATT       1080
```

TAAAGGACAT ACTAATCTGA TCTGTTCAGG CTCTTTTCTC TCAGAGTCCA AGCAC   1135

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 358 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (vi) ORIGINAL SOURCE:
        (F) TISSUE TYPE: Mouse Lung

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Tyr Gly Glu Trp Gly Met Gly Asn Ile Leu Met Met Phe His Val
1               5                   10                  15

Tyr Leu Val Gln Gly Phe Arg Ser Glu His Gly Pro Val Lys Asp Phe
            20                  25                  30

Ser Phe Glu Arg Ser Ser Arg Ser Met Leu Glu Arg Ser Glu Gln Gln
        35                  40                  45

Ile Arg Ala Ala Ser Ser Leu Glu Glu Leu Leu Gln Ile Ala His Ser
    50                  55                  60

Glu Asp Trp Lys Leu Trp Arg Cys Arg Leu Lys Leu Lys Ser Leu Ala
65                  70                  75                  80

Ser Met Asp Ser Arg Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala
                85                  90                  95

Thr Phe Tyr Asp Thr Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln
            100                 105                 110

Arg Thr Gln Cys Ser Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu
            115                 120                 125

Leu Gly Lys Thr Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val
    130                 135                 140

Phe Arg Cys Gly Gly Cys Cys Asn Glu Glu Gly Val Met Cys Met Asn
145                 150                 155                 160

Thr Ser Thr Ser Tyr Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro
                165                 170                 175

Leu Thr Ser Val Pro Glu Leu Val Pro Val Lys Ile Ala Asn His Thr
                180                 185                 190

Gly Cys Lys Cys Leu Pro Thr Gly Pro Arg His Pro Tyr Ser Ile Ile
        195                 200                 205

Arg Arg Ser Ile Gln Thr Pro Glu Glu Asp Glu Cys Pro His Ser Lys
    210                 215                 220

Lys Leu Cys Pro Ile Asp Met Leu Trp Asp Asn Thr Lys Cys Lys Cys
225                 230                 235                 240

Val Leu Gln Asp Glu Thr Pro Leu Pro Gly Thr Glu Asp His Ser Tyr
                245                 250                 255

Leu Gln Glu Pro Thr Leu Cys Gly Pro His Met Thr Phe Asp Glu Asp
                260                 265                 270
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Arg Cys Glu Cys Val Cys Lys Ala Pro Cys Pro Gly Asp Leu Ile Gln
    275   280   285

His Pro Glu Asn Cys Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Ser
    290   295   300

Cys Cys Gln Lys His Lys Ile Phe His Pro Asp Thr Cys Ser Cys Glu
305   310   315   320

Asp Arg Cys Pro Phe His Thr Arg Thr Cys Ala Ser Arg Lys Pro Ala
            325   330   335

Cys Gly Lys His Trp Arg Phe Pro Lys Glu Thr Arg Ala Gln Gly Leu
            340   345   350

Tyr Ser Gln Glu Asn Pro
            355

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 321 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (vi) ORIGINAL SOURCE:
        (F) TISSUE TYPE: Mouse Lung

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Tyr Gly Glu Trp Gly Met Gly Asn Ile Leu Met Met Phe His Val
1   5   10   15

Tyr Leu Val Gln Gly Phe Arg Ser Glu His Gly Pro Val Lys Arg Ser
            20   25   30

Ser Arg Ser Met Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser
        35   40   45

Ser Leu Glu Glu Leu Leu Gln Ile Ala His Ser Glu Asp Trp Lys Leu
    50   55   60

Trp Arg Cys Arg Leu Lys Leu Lys Ser Leu Ala Ser Met Asp Ser Arg
65   70   75   80

Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Thr
            85   90   95

Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser
            100   105   110

Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Thr Thr
        115   120   125

Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly
        130   135   140

Cys Cys Asn Glu Glu Gly Val Met Cys Met Asn Thr Ser Thr Ser Tyr
145   150   155   160

Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro
            165   170   175

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Glu Leu Val Pro Val Lys Ile Ala Asn His Thr Gly Cys Lys Cys Leu
            180                 185                 190

Pro Thr Gly Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln
            195                 200                 205

Thr Pro Glu Glu Asp Glu Cys Pro His Ser Lys Lys Leu Cys Pro Ile
    210                 215                 220

Asp Met Leu Trp Asp Asn Thr Lys Cys Lys Cys Val Leu Gln Asp Glu
225                 230                 235                 240

Thr Pro Leu Pro Gly Thr Glu Asp His Ser Tyr Leu Gln Glu Pro Thr
                245                 250                 255

Leu Cys Gly Pro His Met Thr Phe Asp Glu Asp Arg Cys Glu Cys Val
            260                 265                 270

Cys Lys Ala Pro Cys Pro Gly Asp Leu Ile Gln His Pro Glu Asn Cys
        275             280                 285

Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Ser Cys Cys Gln Lys His
        290             295                 300

Lys Ile Phe His Pro Asp Thr Cys Arg Ser Met Val Phe Ser Leu Ser
305                 310                 315                 320

Pro
```

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nuleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (vi) ORIGINAL SOURCE:
        (F) TISSUE TYPE:

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGCTGCTTC TAGTTTGGAG   20

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nuleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: oligonucleotide

    (vi) ORIGINAL SOURCE:
        (F) TISSUE TYPE:

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACTCGCAAC GATCTTCGTC   20

Patent Claims

1.   An isolated nucleic acid molecule comprising a
nucleic acid sequence which encodes a polypeptide
comprising a sequence of amino acids substantially
5   corresponding to the amino acid sequence set out in SEQ ID
NO.3, SEQ ID NO.5, SEQ ID NO.8 or SEQ ID NO. 9, said
polypeptide having the ability to stimulate vascular
permeability or proliferation of endothelial cells, or a
fragment or analogue thereof which has the ability to
10   stimulate at least one biological activity selected from
the group consisting of angiogenesis, vascular
permeability, endothelial cell proliferation,
differentiation, migration or survival, or which has the
ability to bind to endothelial cells, but is unable to
15   stimulate at least one of said biological activities.

2.   A nucleic acid molecule according to claim 1,
wherein said nucleic acid molecule comprises a nucleic
acid sequence which encodes the amino acid sequence
Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys
5   (SEQ ID NO.2).

3.   A nucleic acid molecule according to claim 1,
wherein said endothelial cells are selected from the group
consisting of vascular endothelial cells and lymphatic
endothelial cells.

4.   A nucleic acid molecule according to claim 1,
which is a genomic DNA.

5.   A nucleic acid molecule according to claim 1,
which is a cDNA.

6.   A nucleic acid molecule according to claim 5,
which comprises the nucleic acid sequence of SEQ ID NO.1,
SEQ ID NO.4, SEQ ID NO.6, SEQ ID NO.7, or a DNA sequence

which hybridizes to one of the foregoing sequences under
5   stringent conditions.

7.   A nucleic acid molecule according to claim 6,
which comprises the nucleic acid sequence of SEQ ID NO.4.

8.   A nucleic acid molecule according to any one of
claims 1 to 7, which encodes a polypeptide which has the
ability to stimulate vascular permeability or
proliferation of endothelial cells.

9.   A nucleic acid molecule according to claim 1,
which encodes a polypeptide comprising amino acid residues
64 through 172 of SEQ ID NO:3 or amino acid residues 93
through 201 of SEQ ID NO:5.

10.   A nucleic acid molecule according to claim 9,
wherein said polypeptide further comprises an affinity tag
peptide sequence.

11.   A nucleic acid molecule according to any one of
Claims 1 to 7, which encodes a polypeptide which has the
ability to bind to endothelial cells but is unable to
stimulate endothelial cell proliferation.

12.   A nucleic acid molecule according to claim 11,
wherein said endothelial cells are selected from the group
consisting of vascular endothelial cells and lymphatic
endothelial cells.

13.   A nucleic acid molecule according to claim 1,
wherein said nucleic acid molecule is a human DNA
molecule.

14.   A vector comprising a nucleic acid according to
any one of Claims 1 to 13.

15. A host cell transformed or transformed with a vector according to claim 14.

16. An isolated polypeptide which comprises a sequence of amino acids substantially corresponding to the amino acid sequence set out in SEQ ID NO.3, SEQ ID NO.5, SEQ ID NO.8 or SEQ ID NO. 9, said polypeptide having the ability to stimulate vascular permeability or proliferation of endothelial cells, or a fragment or analogue thereof which has the ability to stimulate at least one endothelial cell biological activity selected from the group consisting of cell proliferation, cell differentiation, cell migration, cell survival and vascular permeability, or which has the ability to bind to endothelial cells but is unable to stimulate at least one of said biological activities.

17. A polypeptide according to claim 16, wherein said polypeptide comprises the amino acid sequence
    Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys
(SEQ ID NO.2).

18. A polypeptide according to claim 16, wherein said endothelial cells are selected from the group consisting of vascular endothelial cells and lymphatic endothelial cells.

19. A polypeptide according to Claim 16, which comprises a sequence of amino acids substantially corresponding to SEQ ID NO:3 or SEQ ID NO:5.

20. A polypeptide according to Claim 19, which comprises a sequence of amino acids substantially corresponding to SEQ ID NO 5.

21. A polypeptide according to any one of Claims 16 to 20, which has the ability to stimulate proliferation of endothelial cells.

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22.   A polypeptide according to any one of claims 16 to 20, which has the ability to induce endothelial cell differentiation.

23.   A polypeptide according to any one of claims 16 to 20, which has the ability to induce vascular permeability.

24.   A polypeptide according to Claim 16, comprising amino acid residues 64 through 172 of SEQ ID NO:3 or 93 through 201 of SEQ ID NO:5.

25.   A polypeptide according to claim 24, further comprising an affinity tag peptide sequence.

26.   A polypeptide according to claim 16 or 17, which has the ability to bind to endothelial cells but is unable to stimulate proliferation of endothelial cells.

27.   A polypeptide according to claim 26, wherein said endothelial cells are selected from the group consisting of vascular endothelial cells and lymphatic endothelial cells.

28.   A polypeptide according to any one of claims 16 to 20, wherein said polypeptide is a human protein.

29.   An antibody specifically reactive with a polypeptide according to any one of Claims 16 to 28.

30.   An antibody according to claim 29, wherein said antibody is a polyclonal antibody.

31.   An antibody according to claim 29, wherein said antibody is a monoclonal antibody.

32.   An antibody according to claim 29, wherein said antibody is labelled with a detectable label.

33.   A method of making a polypeptide according to
claim 16, said method comprising the steps of:
      culturing a host cell transformed or transfected with
a vector comprising a nucleic acid sequence encoding said
5 polypeptide operably associated with a promoter sequence
such that the nucleic acid sequence encoding said
polypeptide is expressed; and
      isolating said polypeptide from said host cell or
from a growth medium in which said host cell is cultured.

34.   A method of isolation of VEGF-D comprising the
step of exposing a cell which expresses VEGF-D to heparin
to facilitate release of VEGF-D from the cell, and
purifying the thus-released VEGF-D.

35.   A method of making a vector capable of
expressing a polypeptide encoded by a nucleic acid
molecule according to any one of Claims 1 to 9, said
method comprising inserting said nucleic acid molecule
5 into a vector in a position in which said nucleic acid
molecule is operatively connected with at least one
promoter.

36.   A vector comprising an anti-sense nucleotide
sequence, said anti-sense nucleotide sequence being
complementary to at least a part of a VEGF-D genomic DNA
sequence or a VEGF-D RNA sequence or a cDNA sequence which
5 encodes VEGF-D or a fragment or analogue thereof which
promotes at least one bioactivity selected from vascular
permeability, proliferation of endothelial cells and
endothelial cell differentiation, whereby said vector can
be used to inhibit said at least one bioactivity.

37.   A method of stimulating endothelial cell
proliferation comprising contacting endothelial cells with
an effective endothelial cell proliferation stimulating
amount of a polypeptide according to claim.16.

38. A method according to claim 37, wherein said endothelial cells are selected from the group consisting of vascular endothelial cells and lymphatic endothelial cells.

39. A method of stimulating at least one bioactivity selected from endothelial cell proliferation, endothelial cell differentiation and vascular permeability, *in vivo* in a mammal, said method comprising administering to said
5   mammal an effective bioactivity stimulating amount of a polypeptide according to claim 16, which has the ability to stimulate said at least one bioactivity.

40. A method according to Claim 39, wherein said polypeptide comprises amino acid residues 64 through 172 of SEQ ID NO:3 or amino acid residues 93 through 201 of SEQ ID NO:5.

41. A method according to Claim 39, wherein lymphatic vessel endothelial cell proliferation is stimulated.

42. A method of stimulating at least one bioactivity selected from angiogenesis and neovascularization in a mammal, said method comprising the step of administering to said mammal an effective angiogenesis or
5   neovascularization stimulating amount of a polypeptide according to claim 16, said polypeptide having the ability to stimulate endothelial cell proliferation.

43. A method according to Claim 42, wherein said polypeptide comprises amino acid residues 64 through 172 of SEQ ID NO:3 or amino acid residues 93 through 201 of SEQ ID NO:5.

44. A method according to claim 43, wherein said polypeptide further comprises an affinity tag peptide sequence.

45. A method according claim 32, further comprising
co-administering at least one substance selected from the
group consisting of VEGF, VEGF-B, VEGF-C, PlGF, PDGF, FGF
and heparin.

46. A method of inhibiting a bioactivity selected
from angiogenesis and neovascularization in a mammal, said
method comprising the step of administering to said mammal
an effective angiogenesis or neovascularization inhibiting
5   amount of a VEGF-D antagonist.

47. A method according to claim 46, wherein said
VEGF-D antagonist comprises an antibody specific to
VEGF-D.

48. A method according to claim 46, wherein said
VEGF-D antagonist comprises a polypeptide which binds to
endothelial cells but which is unable to stimulate at
least one biological activity selected from proliferation
5   of endothelial cells, endothelial cell differentiation and
vascular permeability.

49. A method according to claim 48, wherein said
endothelial cells are selected from the group consisting
of vascular endothelial cells and lymphatic endothelial
cells.

50. A method of inhibiting VEGF-D expression in a
mammal comprising the step of transforming target cells
expressing VEGF-D with a vector according to Claim 36.

51. A pharmaceutical composition comprising a
polypeptide according to any one of claims 16 to 24, and a
pharmaceutically acceptable carrier or adjuvant.

52. A pharmaceutical composition according to claim 51, further comprising at least one substance selected from the group consisting of VEGF, VEGF-B, VEGF-C, PlGF, PDGF and heparin.

53. A pharmaceutical composition comprising an antibody according to any one of claims 29 through 32, and a pharmaceutically acceptable carrier or adjuvant.

54. A pharmaceutical composition according to Claim 53, wherein said antibody is a monoclonal antibody.

55. A protein dimer comprising a first polypeptide according to any one of claims 16 to 24, and a second polypeptide.

56. A protein dimer according to Claim 55, wherein said protein dimer is a homodimer in which the second polypeptide is identical to the first polypeptide.

57. A protein dimer according to claim 55, wherein said protein dimer is a heterodimer in which the second polypeptide is selected from VEGF, VEGF-B, VEGF-C, PlGF and PDGF.

58. A method of detecting VEGF-D in a biological sample, comprising the step of contacting the sample with a reagent capable of binding VEGF-D, and detecting the occurrence of binding of said reagent.

59. A method according to claim 58, wherein said reagent comprises an antibody according to any one of claims 29 to 32.

60. A method of modulating vascular permeability in a mammal, said method comprising administering to said mammal an effective vascular permeability modulating amount of a polypeptide according to any one of claims 16
5   to 24, or an antibody according to any one of claims 29 to 32.

61. A method according to claim 60, comprising administering to said mammal a polypeptide according to claim 16, having the ability to stimulate endothelial cell proliferation.

62. A method according to claim 60, comprising administering to said mammal a polypeptide according to claim 16, which has the ability to bind to endothelial cells, but which is unable to stimulate endothelial cell
5   proliferation.

63. A method of activation of VEGF receptor 2, comprising the step of exposing cells bearing said receptor to an effective receptor activating dose of VEGF-D.

64. A method of activation of VEGF receptor 3, comprising the step of exposing cells bearing said receptor to an effective receptor activating dose of VEGF-D.

65. A method according to claim 63 or 64, wherein said method is carried out *in vivo*.

66. A method according to claim 63 or 64, wherein said method is carried out *in vitro*.

67.   A diagnostic or prognostic test kit comprising a
specific binding reagent for VEGF-D and means for
detecting binding of said reagent.

68.   A test kit according to claim 67, wherein said
specific binding reagent comprises an antibody according
to any one of claims 29 to 32.

69.   A diagnostic or prognostic test kit comprising a
pair of primers specific to VEGF-D DNA operatively coupled
to a polymerase, whereby said polymerase is enabled to
selectively amplify VEGF-D DNA from a DNA sample.

70.   A method of detecting aberrations in VEGF-D gene
structure in a test subject comprising the steps of:
        providing a DNA sample from said test subject;
        contacting said sample with a set of primers specific
5   to VEGF-D DNA operatively coupled to a polymerase and
selectively amplifying VEGF-D DNA from said sample by
polymerase chain reaction; and
        comparing the nucleotide sequence of the amplified
VEGF-D DNA from said sample with a nucleotide sequence as
10   set forth in SEQ ID NO:1 or SEQ ID NO:4.

71.   A VEGF-D antagonist having the capability to
inhibit at least one biological activity induced by VEGF-D
selected from vascular permeability, endothelial cell
proliferation and endothelial cell differentiation, said
5   antagonist binding to VEGF-D or to a VEGF-D receptor, but
being less able than VEGF-D to stimulate said at least one
biological activity.

72.   A VEGF-D antagonist according to claim 71,
wherein said antagonist comprises an antibody which
selectively binds VEGF-D.

73. A VEGF-D antagonist according to claim 72, wherein said antibody is a monoclonal antibody.

74. A VEGF-D antagonist according to claim 71, wherein said antagonist comprises a VEGF-D polypeptide fragment or analogue which binds to a VEGF-D receptor, but is less able to stimulate said at least one biological

5 activity.

75. A method of improving pulmonary blood circulation and/or gas exchange in a mammal, said method comprising administering to said mammal an effective blood circulation and/or gas exchange improving amount of

5 VEGF-D.

76. A method of treating fluid accumulation in the heart and/or lung due to increases in vascular permeability in a mammal, said method comprising administering to said mammal an effective vascular

5 permeability decreasing amount of a VEGF-D antagonist.

77. A method of treating an intestinal malabsorption syndrome in a patient suffering therefrom, said method comprising administering to said patient an effective intestinal blood circulation and/or vascular permeability

5 increasing amount of VEGF-D.

```
1    M R S S Q S - - - - T L E R S E Q Q I R A A S S L E E L L R   VEGF-D
1    M N F L L S W V H W S L A - - - - - - - - - - - - - L L L     h VEGF 165

27   I T H S E D W K L W R C R L R L K S F T S M D S R S A S H R   VEGF-D
17   Y L H H A K W S - - - - - - - Q A A P M A E G G G Q N H H     h VEGF 165

57   S T - R F A A T F Y D I E T L K V I D E E W Q R T Q C S P R   VEGF-D
39   E V V K F M D V Y - - - - - - - - - - - - - Q R S Y C H P I   h VEGF 165

86   E T C V E V A S E L G K S T N T F F K P P C V N V F R C G G   VEGF-D
56   E T L V D I F Q E Y P D E I E Y I F K P S C V P L M R C G G   h VEGF 165

116  C C N E E S L I C M N T S T S Y I S K Q L F E I S V P L T S   VEGF-D
86   C C N D E G L E C V P T E E S N I T M Q I M R I K P - - H Q   h VEGF 165

146  V P E L V P V K V A N H T G C K C L P T A P R H P Y S I I R   VEGF-D
114  G Q H I G E M S F L Q H N K C E C R P K K D R - - - - - - -   h VEGF 165

176  R S I Q I P E E D R C S H S K K L C P I D M L W D S N K C K   VEGF-D
137  - - - - A R Q E N P C G P C - - - - - - - - - - - - - - - -   h VEGF 165

206  C V L Q E E N P L A G T E D H S H L Q E P A L C G P H M M F   VEGF-D
147  - - - - - - - - - S E R R K H L - - - - - - - - - - - - - -   h VEGF 165

236  D E D R C E C V C K T P C P K D L I Q H P K N C S C F E C K   VEGF-D
154  - - - - - - - - - - - - - F V Q D P Q T C K C - S C K       h VEGF 165

266  E S L E T C C Q K H K L F H P D T C S C E D R C P F H T R P   VEGF-D
167  N T D S R C K A R Q L E L N E R T C R C D - - - - - - - - -   h VEGF 165

296  C A S G K T A C A K H C R F P K E K R A A Q G P H S R K N P   VEGF-D
188  - - - - - - - - - - - - - - - - - - - - - - - K P R R       h VEGF 165
```

FIG. 1a

```
 1   M R S S Q S T L E R S E Q Q I R A A S S L E E L L R I T H S    VEGF-D
 1   M S P L L R R L - - - - - - - - - - - - L L A A L L Q L A P A  h VEGF-B

31   E D W K L W R C R L R L K S F T S M D S R S A S H R S T R F    VEGF-D
20   Q A - - - - - - - - - - - P V S Q P D A P G H Q R K V V S W    h VEGF-B

61   A A T F Y D I E T L K V I D E E W Q R T Q C S P R E T C V E    VEGF-D
39   I D V Y - - - - - - - - - - - - T R A T C Q P R E V V V P    h VEGF-B

91   V A S E L G K S T N T F F K P P C V N V F R C G G C C N E E    VEGF-D
56   L T V E L M G T V A K Q L V P S C V T V Q R C G G C C P D D    h VEGF-B

121  S L I C M N T S T S Y I S K Q L F E I S V P L T S V P E L V    VEGF-D
86   G L E C V P T G Q H Q V R M Q I L M I R Y P S S Q L G E M -    h VEGF-B

151  P V K V A N H T G C K C L P T A P R H P Y S I I R R S I Q I    VEGF-D
115  - - S L E E H S Q C E C R P K - - - - - - - - K K D S A V    h VEGF-B

181  P E E D R C S H S K K L C P I D M L W D S N K C K C V L Q E    VEGF-D
134  K P D S - - - - P R P L C P - - - - - - - - - R C T Q H H    h VEGF-B

211  E N P L A G T E D H S H L Q E P A L C G P H M M F D E D R C    VEGF-D
150  Q R P - - - - - - - - - - - - - - - - - - D P R T C    h VEGF-B

241  E C V C K T P C P K D L I Q H P K N C S C F E C K E S L E T    VEGF-D
158  R C R C R R - - - R S F L R - - - - - - - - - - - - -    h VEGF-B

271  C C Q K H K L - F H P D T C S C E D R C P F H T R P C A S G    VEGF-D
169  - C Q G R G L E L N P D T C R C - - - - - - - - - - - -    h VEGF-B

300  K T A C A K H C R F P K E K R A A Q G P H S R K N P    VEGF-D
184  - - - - - - - - - - - - - - - - - - - - R K L R R    h VEGF-B
```

FIG. 1b

```
1    M R S S Q S T L E R S E Q Q I R A A S S L E E L L R I T H S   VEGF-D
1    M T - - - - - - - - - - - - - - - - - - - - - V L Y P   VEGF-C

31   E D W K L W R C R L R - - - - - L K S F T S M D S R S A S   VEGF-D
7    E Y W K M Y K C Q L R K G G W Q H N R E Q A N L N S R T E E   VEGF-C

55   H R S T R F A A T F Y D I E T L K V I D E E W Q R T Q C S P   VEGF-D
37   - - T I K F A A A H Y N T E I L K S I D N E W R K T Q C M P   VEGF-C

85   R E T C V E V A S E L G K S T N T F F K P P C V N V F R C G   VEGF-D
65   R E V C I D V G K E F G V A T N T F F K P P C V S V Y R C G   VEGF-C

115  G C C N E E S L I C M N T S T S Y I S K Q L F E I S V P L T   VEGF-D
95   G C C N S E G L Q C M N T S T S Y L S K T L F E I T V P L S   VEGF-C

145  S V P E L V P V K V A N H T G C K C L P T A P - - R H P Y S   VEGF-D
125  Q G P K P V T I S F A N H T S C R C M S K L D V Y R Q V H S   VEGF-C

173  I I R R S I Q I P E E D R C S H S K K L C P I D M L W D S N   VEGF-D
155  I I R R S L P A T L P Q - C Q A A N K T C P T N Y M W N N H   VEGF-C

203  K C K C V L Q E E - - - N P L A G T E D - - - - - - - - -   VEGF-D
184  I C R C L A Q E D F M F S S D A G D D S T D G F H D I C G P   VEGF-C

220  H S H L Q E - - - - - - - - - - - - - - - - - - - - - -   VEGF-D
214  N K E L D E E T C Q C V C R A G L R P A S C G P H K E L D R   VEGF-C

226  - - - - - - - - - P A L C G P H M M F D E D R C E C V   VEGF-D
244  N S C Q C V C K N K L F P S Q C G A N R E F D E N T C Q C V   VEGF-C

244  C K T P C P K D L I Q H P K N C S C F E C K E S L E T C C Q   VEGF-D
274  C K R T C P R N Q P L N P G K C A C - E C T E S P Q K C L L   VEGF-C

274  K H K L F H P D T C S C E D R C P F H T R P C A S G K T A C   VEGF-D
303  K G K K F H H Q T C S C - - - - - - Y R R P C T N R Q K A C   VEGF-C

304  A K H C R F P K E K - R A A Q G P H S R K N P .   VEGF-D
327  E P G F S Y S E E V C R C V P S Y W K R R Q M S   VEGF-C
```

FIG. 1c

```
1   M R S S Q - - - - - - - - S T L E R S E Q Q I R A A S S L   VEGF-D
1   M P V M R L F P C F L Q L L A G L A - - - - - - - - - - -   hPlGF

22  E E L L R I T H S E D W K L W R C R L R L K S F T S M D S R   VEGF-D
19  - - - L P A V P P Q Q W A L - - - - - - - - - - - - - - -   hPlGF

52  S A S H R S T R F A A T F Y D I E T L K V I D E E W Q R T Q   VEGF-D
30  S A G N G S S - - - - - - - E V E V V P - F Q E V W G R S Y   hPlGF

82  C S P R E T C V E V A S E L G K S T N T F F K P P C V N V F   VEGF-D
52  C R A L E R L V D V V S E Y P S E V E H M F S P S C V S L L   hPlGF

112 R C G G C C N E E S L I C M N T S T S Y I S K Q L F E I S V   VEGF-D
82  R C T G C C G D E N L H C V P V E T A N V T M Q L L K I R S   hPlGF

142 P L T S V P E L V P V K V A N H T G C K C L P T A P R H P Y   VEGF-D
112 - - G D R P S Y V E L T F S Q H V R C E C R P - - - - - - -   hPlGF

172 S I I R R S I Q I P E E D R C S H S K K L C P I D M L W D S   VEGF-D
133 - - L R E K M K - P E R R R - - - - - - - - - - - - - - -   hPlGF

202 N K C K C V L Q E E N P L A G T E D H S H L Q E P A L C G P   VEGF-D
144 - - - - - - - - - - - - - - - - - - - - - - - - - - - -   hPlGF

232 H M M F D E D R C E C V C K T P C P K D L I Q H P K N C S C   VEGF-D
144 - - - - - - - - - - - - - - - - - - - - - - - - - - - -   hPlGF

262 F E C K E S L E T C C Q K H K L F H P D T C S C E D R C P F   VEGF-D
144 - - - - - - - - - - - - - - - - - - - - - - - - - - - -   hPlGF

292 H T R P C A S G K T A C A K H C R F P K E K R A A Q G P - -   VEGF-D
144 - - - - - P K G R G - - - - - - - - - K R R R E K Q R P T D   hPlGF

320 - - - - - H S R K N P                                        VEGF-D
160 C H L C G D A V P R R                                        hPlGF
```

FIG. 1d

FIG. 2

```
  1  M R S S Q S T L E R S E Q Q I R A A S S L E E L L R I T H S        VEGF-D
  1  M T - - - - - - - - - - - - - - - - - - - - - - - - V L Y P        VEGF-C
  1  M S - - - - - - - - - - - - - - - - - - - - - - P L - - - -        h VEGF-B
  1  M - - - - - - - - - - - - - - - - - - - - - - - N F L L S          h VEGF 165
  1  M P - - - - - - - - - - - - - - - - - - - - V M R L F P C          hPlGF

 31  E D W K L H R C R L R L K S F - - - - - - T S M D S R S A          VEGF-D
  7  E Y W K M Y K Q L R K G G W Q R - N R E Q A N L N S R T -          VEGF-C
  5  - - - - - - L R R L L A A L L Q L A P A Q A P V S Q P D A          h VEGF-B
  7  - - W V H W S L A L L - Y L H H A K W S Q A A P M A E G G          h VEGF 165
 10  - - F L Q L L A G L A L P A V P P Q Q W A - - - - L S A G N        hPlGF

 54  S H R S T R F A A T F Y D I E T L K V I D E E W Q R T Q C S        VEGF-D
 35  - E E T I K F A A A H Y N T E I L K S I D N E H R K T Q C M        VEGF-C
 29  P G H - - - - - - - - Q R K V V S W I D V - Y T R A T C Q          h VEGF-B
 34  G Q N - - - - - - - - H R E V V K F M D V - Y Q R S Y C H          h VEGF 165
 34  G S S - - - - - - - - E V E V V P F Q E V - H G R S Y C R          hPlGF

 84  P R E T C V E V A S E L G K S T N T F F K P P C V N V F R C        VEGF-D
 64  P R E V C I D V G K E F G V A T N T F F K P P C V S V Y R C        VEGF-C
 49  P R E V V V P L T V E L M G T V A K Q L V P S C V T V Q R C        h VEGF-B
 54  P I E T L V D I F Q E Y P D E I E Y I F K P S C V P L M R C        h VEGF 165
 54  A L E R L V D V V S E Y P S E V E H M F S P S C V S L L R C        hPlGF

114  G G C C N E E S L I C M N T S T S Y I S K Q L F E I S V P L        VEGF-D
 94  G G C C N S E G L Q C M N T S T S Y L S K T L F E I T V P L        VEGF-C
 79  G G C C P D D G L E C V P T G Q H Q V R M Q I L M I R - - -        h VEGF-B
 84  G G C C N D E G L E C V P T E E S N I T M Q I M R I K P - -        h VEGF 165
 84  T G C C G D E N L H C V P V E T A N V T M Q L L K I R S - -        hPlGF

144  T S V P E L V P V K V A N E T G C K C L P T A P - - R H P Y        VEGF-D
124  S Q G P K P V T I S F A N H T S C R C M S K L D V Y R Q V H        VEGF-C
106  Y P S S Q I G E M S L E E H S Q C E C - - - - - - - - - -        h VEGF-B
112  H Q G Q H I G E M S F L Q H N K C E C - - - - - - - - - -        h VEGF 165
112  G D R P S Y V E L T F S Q H V R C E C - - - - - - - - - -        hPlGF

172  S I I R R S I Q I P E E D R C S H S K K L C P I D M L W D S        VEGF-D
154  S I I R R S L P - A T L P Q C Q A A N K T C P T N Y H W N N        VEGF-C
125  - - - - R P K K K D S A V K P D S P R P L C P - - - - - -        h VEGF-B
131  - - - - R P K K D R - - A R Q E N P - - C G - - - - - - -        h VEGF 165
131  - - - - R P L R E K - - M K - - - - - - - - - - - - - - -        hPlGF

202  N K C K C V L Q E E N P L A G T E D - - - - - - - - - - -        VEGF-D
183  H I C R C L A Q E D F M F S S D A G D D S T D G F H D I C G        VEGF-C
144  - - - R C T Q H Q R - - - P - - - - - - - - - - - - - - -        h VEGF-B
145  - - - P C S E R R K H L F V Q - - - - - - - - - - - - - -        h VEGF 165
139  - - - - - P E R R R P - - - - - - - - - - - - - - - - - -        hPlGF

220  - H S H L Q E - - - - - - - - - - - - - - - - - - - - - -        VEGF-D
213  P N K E L D R E T C Q C V C R A G L R P A S C G P H K E L D        VEGF-C
153  - - - - - - - - - - - - - - - - - - - - - - - - - - - - D        h VEGF-B
157  - - - - - - - - - - - - - - - - - - - - - - - - - - - - D        h VEGF 165
145  - - - - - - - - - - - - - - - - - - - - - - - - - - - - -        hPlGF

226  - - - - - - - - - - - - P A L C G P H M M F D E D R C E C        VEGF-D
243  R N S C Q C V C K N K L F P S Q C G A N R E F D E N T C Q C        VEGF-C
154  P R T C R C R C R R R S F - - - - - - - - - - - - - - - -        h VEGF-B
158  P Q T C K C S C K N T D - - - - - - - - - - - - - - - - -        h VEGF 165
145  - - - - - - K G R G - - - - - - - - - - - - - - - - - - -        hPlGF

243  V C K T P C P K D L I Q H P K N C S C F E C K E S L E T C C        VEGF-D
273  V C K R T C P R N Q P L N P G K C A C - E C T E S P Q K C L        VEGF-C
167  - - - - - - - - - - - - - - - - - - - - - - - - L R C Q          h VEGF-B
170  - - - - - - - - - - - - - - - - - - - - - - - - S R C K          h VEGF 165
149  - - - - - - - - - - - - - - - - - - - - - - - - K R R R          hPlGF

273  Q K H K L F H P D T C S C E D R - C P F H T R P C A S G K T        VEGF-D
302  L N G K K F H Q T C S C Y R R P C T N R Q K A C E P G F S          VEGF-C
171  G R G L E L N P D T C R C - - - - - - - - - - - - - - - -        h VEGF-B
174  A R Q L E L N E R T C R C - - - - - - - - - - - - - - - -        h VEGF 165
153  E K Q R P T D C H L C G D - - - - - - - - - - - - - - - -        hPlGF

302  A C A K H C R F P K F K R A A Q G P H S R K N P                    VEGF-D
332  Y S E E V C R C V P S Y W - - - - - K R R Q M S                    VEGF-C
184  - - - - - - - - - - - - - - - - - - R K L R R                      h VEGF-B
187  - - - - - - - - - - - - - - - - - - D K P R R                      h VEGF 165
166  - - - - - - - - - - - - - - - - - - A V P R R                      hPlGF
```

FIG. 3

```
        10          20          30          40
MRSSQSTLERSEQQIRAASSLEELLRITHSEDWKLWRCRL


        50          60          70          80
RLKSFTSMDSRSASHRSTRFAATFYDIETLKVIDEEWQRT


        90         100         110         120
QCSPRETCVEVASELGKSTNTFFKPPCVNVFRCGGCCNEE


       130         140         150         160
SLICMNTSTSYISKQLFEISVPLTSVPELVPVKVANHTGC


       170         180         190         200
KCLPTAPRHPYSIIRRSIQIPEEDRCSHSKKLCPIDMLWD


       210         220         230         240
SNKCKCVLQEENPLAGTEDHSHLQEPALCGPHMMFDEDRC


       250         260         270         280
ECVCKTPCPKDLIQHPKNCSCFECKESLETCCQKHKLFHP


       290         300         310         320
DTCSCEDRCPFHTRPCASGKTACAKHCRFPKEKRAAQGPH


SRKNP
```

FIG. 4

GTTGGGGTTCCAGCTTTCTGTAGCTGTAAGCATTGGTGGCCACACCACCTCCTTACAA
AGCAACTAGAACCTGCGGCATACATTGGAGAGATTTTTTTTAATTTTCTGGACATGAA
GTAAATTTAGAGTGCTTTCTAATTTCAGGTAGAAGACATGTCCACCTTCTGATTATT
TTTGGAGAACATTTTGATTTTTTTCATCTCTCTCTCCCCACCCCTAAGATTGTGCAA
AAAAAGCGTACCTTGCCTAATTGAAATAATTTCATTGGATTTTGATCAGAACTGATT
ATTTGGTTTTCTGTGTGAAGTTTTGAGGTTTCAAACTTTCCTTCTGGAGAATGCCTT
TTGAAACAATTTTCTCTAGCTGCCTGATGTCAACTGCTTAGTAATCAGTGGATATTG
AAATATTCAAAATGTACAGAGAGTGGGTAGTGGTGAATGTTTTCATGATGTTGTACG
TCCAGCTGGTGCAGGGCTCCAGTAATGAACATGGACCAGTGAAGCGATCATCTCAGT
CCACATTGGAACGATCTGAACAGCAGATCAGGGCTGCTTCTAGTTTGGAGGAACTAC
TTCGAATTACTCACTCTGAGGACTGGAAGCTGTGGAGATGCAGGCTGAGGCTCAAAA
GTTTTACCAGTATGGACTCTCGCTCAGCATCCCATCGGTCCACTAGGTTTGCGGCAA
CTTTCTATGACATTGAAACACTAAAAGTTATAGATGAAGAATGGCAAAGAACTCAGT
GCAGCCCTAGAGAAACGTGCGTGGAGGTGGCCAGTGAGCTGGGGAAGAGTACCAACA
CATTCTTCAAGCCCCCTTGTGTGAACGTGTTCCGATGTGGTGGCTGTTGCAATGAAG
AGAGCCTTATCTGTATGAACACCAGCACCTCGTACATTTCCAAACAGCTCTTTGAGA
TATCAGTGCCTTTGACATCAGTACCTGAATTAGTGCCTGTTAAAGTTGCCAATCATA
CAGGTTGTAAGTGCTTGCCAACAGCCCCCCGCCATCCATACTCAATTATCAGAAGAT
CCATCCAGATCCCTGAAGAAGATCGCTGTTCCCATTCCAAGAAACTCTGTCCTATTG
ACATGCTATGGGATAGCAACAAATGTAAATGTGTTTTGCAGGAGGAAAATCCACTTG
CTGGAACAGAAGACCACTCTCATCTCCAGGAACCAGCTCTCTGTGGGCCACACATGA
TGTTTGACGAAGATCGTTGCGAGTGTGTCTGTAAAACACCATGTCCCAAAGATCTAA
TCCAGCACCCCAAAAAACTGCAGTTGCTTTGAGTGCAAAGAAAGTCTGGAGACCTGCT
GCCAGAAGCACAAGCTATTTCACCCAGACACCTGCAGCTGTGAGGACAGATGCCCCT
TTCATACCAGACCATGTGCAAGTGGCAAAACAGCATGTGCAAAGCATTGCCGCTTTC
CAAAGGAGAAAAGGGCTGCCCAGGGGCCCCACAGCCGAAAGAATCCTTGATTCAGCG
TTCCAAGTTCCCCATCCCTGTCATTTTTAACAGCATGCTGCTTTGCCAAGTTGCTGT
CACTGTTTTTTTCCCAGGTGTTAAAAAAAAAAATCCATTTTACACAGCACCACAGTGA
ATCCAGACCAACCTTCCATTCACACCAGCTAAGGAGTCCCTGGTTCATTGATGGATG
TCTTCTAGCTGCAGATGCCTCTGCGCACCAAGGAATGGAGAGGAGGGGACCCATGTA
ATCCTTTTGTTTAGTTTTGTTTTTGTTTTTTGGTGAATGAGAAAGGTGTGCTGGTCA
TGGAATGGCAGGTGTCATATGACTGATTACTCAGÁGCAGATGAGGAAAACTGTAGTC
TCTGAGTCCTTTGCTAATCGCAACTCTTGTGAATTATTCTGATTCTTTTTTATGCAG
AATTTGATTCGTATGATCAGTACTGACTTTCTGATTACTGTCCAGCTTATAGTCTTC
CAGTTTAATGAACTACCATCTGATGTTTCATATTTAAGTGTATTTAAAGAAAATAAA
CACCATTATTCAAGCCAAAAAAAAAAAAAAAAAAAA

MYREWVVVNVFMMLYVQLVQGSSNEHGPVKRSSQSTLERSEQQIRAASSLEELLRIT
HSEDWKLWRCRLRLKSFTSMDSRSASHRSTRFAATFYDIETLKVIDEEWQRTQCSPR
ETCVEVASELGKSTNTFFKPPCVNVFRCGGCCNEESLICMNTSTSYISKQLFEISVP
LTSVPELVPVKVANHTGCKCLPTAPRHPYSIIRRSIQIPEEDRCSHSKKLCPIDMLW
DSNKCKCVLQEENPLAGTEDHSHLQEPALCGPHMMFDEDRCECVCKTPCPKDLIQHP
KNCSCFECKESLETCCQKHKLFHPDTCSCEDRCPFHTRPCASGKTACAKHCRFPKEK
RAAQGPHSRKNP

FIG. 5

FIG. 6

```
GGAGAATGCCTTTTGCAACACTTTTCAGTAGCTGCCTGGAAACAACTGCTTAGTCAT
CGGTAGACATTTAAAATATTCAAAATGTATGGAGAATGGGGAATGGGGAATATCCTC
ATGATGTTCCATGTGTACTTGGTGCAGGGCTTCAGGAGCGAACATGGACCAGTGAAG
GATTTTTCTTTTGAGCGATCATCCCGGTCCATGTTGGAACGATCTGAACAACAGATC
CGAGCAGCTTCTAGTTTGGAGGAGTTGCTGCAAATCGCGCACTCTGAGGACTGGAAG
CTGTGGCGATGCCGGTTGAAGCTCAAAAGTCTTGCCAGTATGGACTCACGCTCAGCA
TCCCATCGCTCCACCAGATTTGCGGCAACTTTCTATGACACTGAAACACTAAAAGTT
ATAGATGAAGAATGGCAGAGGACCCAATGCAGCCCTAGAGAGACATGCGTAGAAGTC
GCCAGTGAGCTGGGGAAGACAACCAACACATTCTTCAAGCCCCCCTGTGTAAATGTC
TTCCGGTGTGGAGGCTGCTGCAACGAAGAGGGTGTGATGTGTATGAACACAAGCACC
TCCTACATCTCCAAACAGCTCTTTGAGATATCAGTGCCTCTGACATCAGTGCCCGAG
TTAGTGCCTGTTAAAATTGCCAACCATACGGGTTGTAAGTGCTTGCCCACGGGCCCC
CGCCATCCTTACTCAATTATCAGAAGATCCATTCAGACCCCAGAAGAAGATGAATGT
CCTCATTCCAAGAAACTCTGTCCTATTGACATGCTGTGGGATAACACCAAATGTAAA
TGTGTTTTGCAAGACGAGACTCCACTGCCTGGGACAGAAGACCACTCTTACCTCCAG
GAACCCACTCTCTGTGGACCGCACATGACGTTTGATGAAGATCGCTGTGAGTGCGTC
TGTAAAGCACCATGTCCGGGAGATCTCATTCAGCACCCGGAAAACTGCAGTTGCTTT
GAGTGCAAAGAAAGTCTGGAGAGCTGCTGCCAAAAGCACAAGATTTTTCACCCAGAC
ACCTGCAGCTGTGAGGACAGATGTCCTTTTCACACCAGAACATGTGCAAGTAGAAAG
CCAGCCTGTGGAAAGCACTGGCGCTTTCCAAAGGAGACAAGGGCCCAGGGACTCTAC
AGCCAGGAGAACCCTTGATTCAACTTCCTTTCAAGTCCCCCCATCTCTGTCATTTTA
AACAGCTCACTGCTTTGTCAAGTTGCTGTCACTGTTGCCCACTACCCCTTGAACATG
TGCAAACACAGACACACACACACACACACAGAGCAACTAGAATTATGTTTTCT
AGGTGCTGCCTAAG
```

FIG. 7

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AAACTTTGCTTCTGGAGAATGCCTTTTGCAACACTTTTCAGTAGCTGCCTGGAAACA
ACTGCTTAGTCATCGGTAGACATTTAAAATATTCAAAATGTATGGAGAATGGGGAAT
GGGGAATATCCTCATGATGTTCCATGTGTACTTGGTGCAGGGCTTCAGGAGCGAACA
TGGACCAGTGAAGCGATCATCCCGGTCCATGTTGGAACGATCTGAACAACAGATCCG
AGCAGCTTCTAGTTTGGAGGAGTTGCTGCAAATCGCGCACTCTGAGGACTGGAAGCT
GTGGCGATGCCGGTTGAAGCTCAAAAGTCTTGCCAGTATGGACTCACGCTCAGCATC
CGATCGCTCCACCAGATTTGCGGCAACTTTCTATGACACTGAAACACTAAAAGTTAT
AGATGAAGAATGGCAGAGGACCCAATGCAGCCCTAGAGAGACATGCGTAGAAGTCGC
CAGTGAGCTGGGGAAGACAACCAACACATTCTTCAAGCCCCCCTGTGTAAATGTCTT
CCGGTGTGGAGGCTGCTGCAACGAAGAGGGTGTGATGTGTATGAACACAAGCACCTC
CTACATCTCCAAACAGCTCTTTGAGATATCAGTGCCTCTGACATCAGTGCCCGAGTT
AGTGCCTGTTAAAATTGCCAACCATACGGGTTGTAAGTGCTTGCCCACGGGCCCCCG
CCATCCTTACTCAATTATCAGAAGATCCATTCAGACCCCAGAAGAAGATGAATGTCC
TCATTCCAAGAAACTCTGTCCTATTGACATGCTGTGGGATAACACCAAATGTAAATG
TGTTTTGCAAGACGAGACTCCACTGCCTGGGACAGAAGACCACTCTTACCTCCAGGA
ACCCACTCTCTGTGGACCGCACATGACGTTTGATGAAGATCGCTGTGAGTGCGTCTG
TAAAGCACCATGTCCGGGAGATCTCATTCAGCACCCGGAAAACTGCAGTTGCTTTGA
GTGCAAAGAAAGTCTGGAGAGCTGCTGCCAAAAGCACAAGATTTTTCACCCAGACAC
CTGCAGGTCAATGGTCTTTTCGCTTTCCCCTTAACTTGGTTTACTGATGACATTTAA
AGGACATACTAATCTGATCTGTTCAGGCTCTTTTCTCTCAGAGTCCAAGCAC
```

FIG. 8

FIG. 9

```
1   M Y R E W V V V N V F M M - - - - L Y V Q L V Q G S S N E H G P V K - - - - -   hVEGF-D
1   M H L L G F F S V A C S L - - - - L A A A L P G P R E A P A A A A F E S G       hVEGF-C
1   M N F L - - - - L S W V H W S L A L L L Y L H H A K W S Q A A B M A - - - -     hVEGF 165
1   M S P L - - - - L R R L - - - - L L A A L L Q L A P A Q A P V S Q - - - -       hVEGF-B
1   M P V M R L F P C F L Q L - - - - L A G L A L P A V P P Q Q W A L S - - - - -   hPlGF

31  - - - - - - - - - - - - - - R S S Q S T L E R S E Q Q I R A A S S L E E L L R I T H S   hVEGF-D
36  L D L S D A E P D A G E A T A Y A S K D L E E Q L R S V L S S V D E L M T V L Y P       hVEGF-C
31  - - - - - - - - - - - - E G G G Q N H H E V V - - - - - - - - - - - - - - -           hVEGF 165
26  - - - - - - - - - - - - P D A P G H Q R K V V - - - - - - - - - - - - - - -           hVEGF-B
31  - - - - - - - - - - - - A G N G S S E V E V V - - - - - - - - - - - - - - -           hPlGF

60  E D W K L W R C R L R - - - L K S F T S M D S R S A S H R S T R F A A T F Y D         hVEGF-D
76  E Y W K M Y K C Q L R K G G W Q H N R E Q A N L N S R T E E T I K F A A A H Y N         hVEGF-C
42  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -           hVEGF 165
37  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -           hVEGF-B
42  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -           hPlGF

96  I E T L K V I D E E W Q R T Q C S P R E T C V E V A S E L G K S T N T F F K P P       hVEGF-D
116 T E I L K S I D N E W R K T Q C M P R E V C I D V G K E F G V A T N T F F K P P       hVEGF-C
42  - - - - - K F M D V Y Q R S Y C H P I E T L V D I F Q E Y P D E I E Y I F K P S       hVEGF 165
37  - - - - - S W I D V Y T R A T C Q P R E V V V P L T V E M G T V A K Q L V P S       hVEGF-B
42  - - - - - P F Q E V W G R S Y C R A L E R L V D V V S E Y P S E V E H M F S P S       hPlGF

136 C V N V F R C G G C C N E E S L I C M N T S T S Y I S K Q L F E I S V P L T S V       hVEGF-D
156 C V S V Y R C G G C C N S E G L Q C M N T S T S Y L S K T L F E I T V P L S Q G       hVEGF-C
77  C V P L M R C G G C C N D E G L E C V P T E E S N I T M Q I M R I K P - - H Q G       hVEGF 165
72  C V T V Q R C G G C C P D D G L E C V P T G Q H Q V R M Q I L M I R - - - Y P S       hVEGF-B
77  C V S L L R C T G C C G D E N L H C V P V E T A N V T M Q L L K I R S - - G D R       hPlGF

176 P E L V P V K V A N H T G C K C L P T A P - - R H P Y S I I R R - - S I Q I P E       hVEGF-D
196 P K P V T I S F A N H T S C R C M S K L D V Y R Q V H S I I R R - - S L P A T L       hVEGF-C
115 Q H I G E M S F L Q H N K C E C R P K K - - R A - - - - - - - R Q E N P C           hVEGF 165
109 S Q L G E M S L E E H S Q C E C R P K K K - - D S - - - - - - A V K P D S P R         hVEGF-B
115 P S Y V E L T F S Q H V R C E C R P L R E - K M - - - - - - - K P E R R R           hPlGF

212 E D R C S H S K K L C P I D M L W D S N K C K C V L Q E E N P L A G T E D H S H       hVEGF-D
234 P Q - C Q A A N K T C P T N Y M W N N H I C R C L A Q E D F M F S S D A G D D S       hVEGF-C
144 G P - C S E R R K - - - - - - - - - - - - - - - - - - - - - - - - - - - - -         hVEGF 165
140 P L - C P R C T Q - - - - - - - - - - - - - - - - - - - - - - - - - - - - -         hVEGF-B
144 P - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -         hPlGF

252 L Q E - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -         hVEGF-D
273 T D G F H D I C G P N K E L D E E T C Q C V C R A G L R P A S C G P H K E L D R       hVEGF-C
152 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -         hVEGF 165
148 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -         hVEGF-B
145 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -         hPlGF

255 - - - - - - - - - P A L C G P H M M F D E D R C E C V C K T P C P K D L I           hVEGF-D
313 N S C Q C V C K N K L F P S Q C G A N R E F D E N T C Q C V C K R T C P R N Q P       hVEGF-C
152 - - - - - - - - - - - - - - - - - - - - - - - - - - - - H L F V                     hVEGF 165
148 - - - - - - - - - - - - - - - - - - - - - - - - - - - - H H Q R                     hVEGF-B
145 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -                   hPlGF

283 Q H P K N C S C F E C K E S L - E T C C Q K H K L F H P D T C S C E D R C P F H       hVEGF-D
353 L N P G K C A C - E C T E S P - Q K C L L K G K K F H Q T C S C - - - - - Y         hVEGF-C
156 Q D P Q T C K C - S C K N T D - S R C K A R Q L E L N E R T C R C - - - - - D         hVEGF 165
152 P D P Q T C R C - R C R R R S F L R C Q G R G L E L N P D T C R C - - - - - R         hVEGF-B
145 - - - K G R G - K R R R E K Q R P T D C H L C G D - - - - - A                     hPlGF

322 T R P C A S G K T A C A K H C R F P K E - - - - - - - - - - K R A A Q G P H S R N     hVEGF-D
385 R R P C T N R Q K A C E P G F S Y S E E V C R C V P S Y W K R P Q M S                 hVEGF-C
188 K - - - - - - - - - - - - - - - - - - - - - - - - - - - - P R R                     hVEGF 165
185 K - - - - - - - - - - - - - - - - - - - - - - - - - - - - L R R                     hVEGF-B
167 V - - - - - - - - - - - - - - - - - - - - - - - - - - - - P R R                     hPlGF

353 N P                                                                                 hVEGF-D
419                                                                                     hVEGF-C
191                                                                                     hVEGF 165
188                                                                                     hVEGF-B
170                                                                                     hPlGF
```

FIG. 10

FIG. 11   VEGFR2 bioassay. Testing of COS cell conditioned medium containing VEGF-A, VEGF-D(Human)
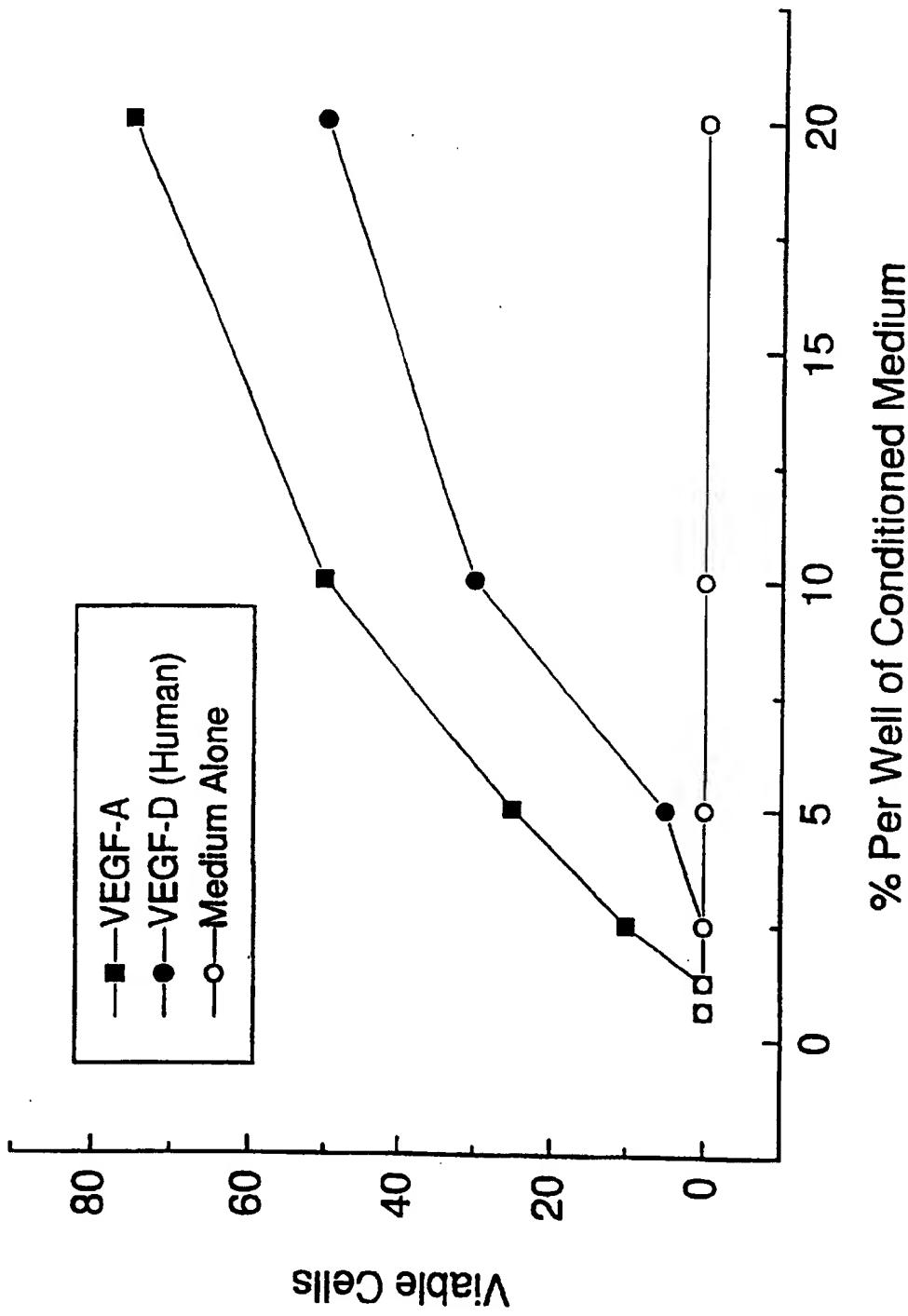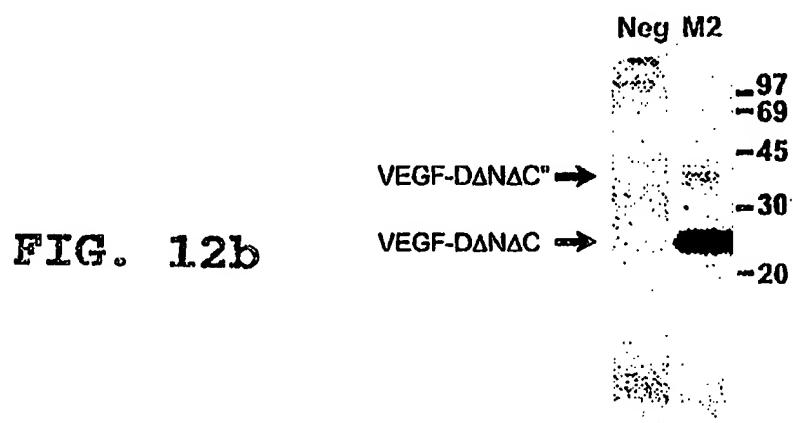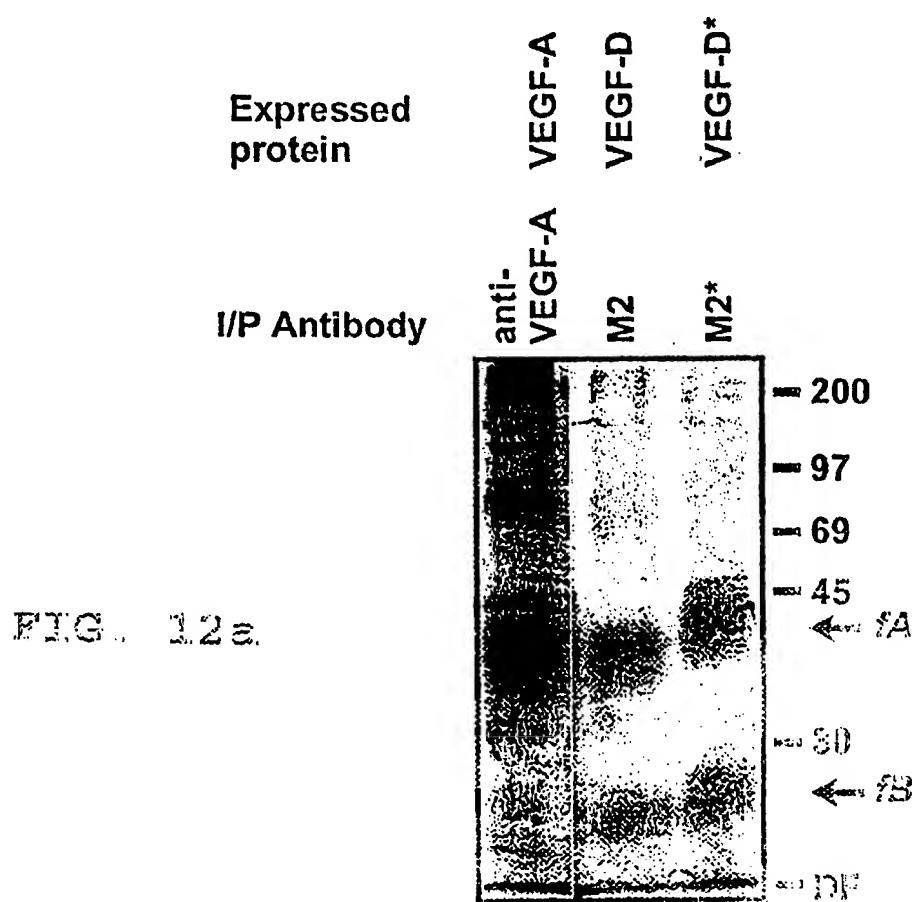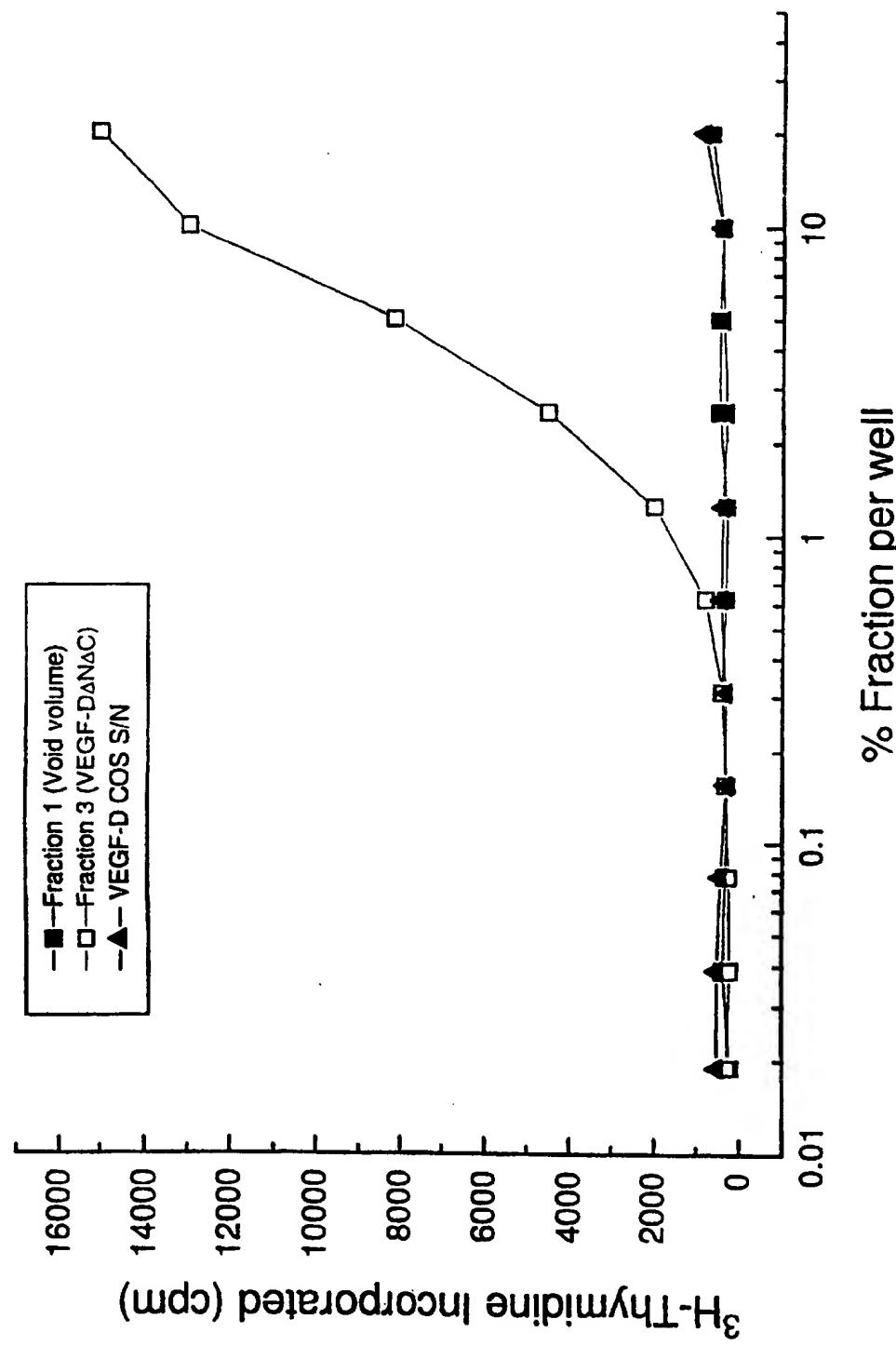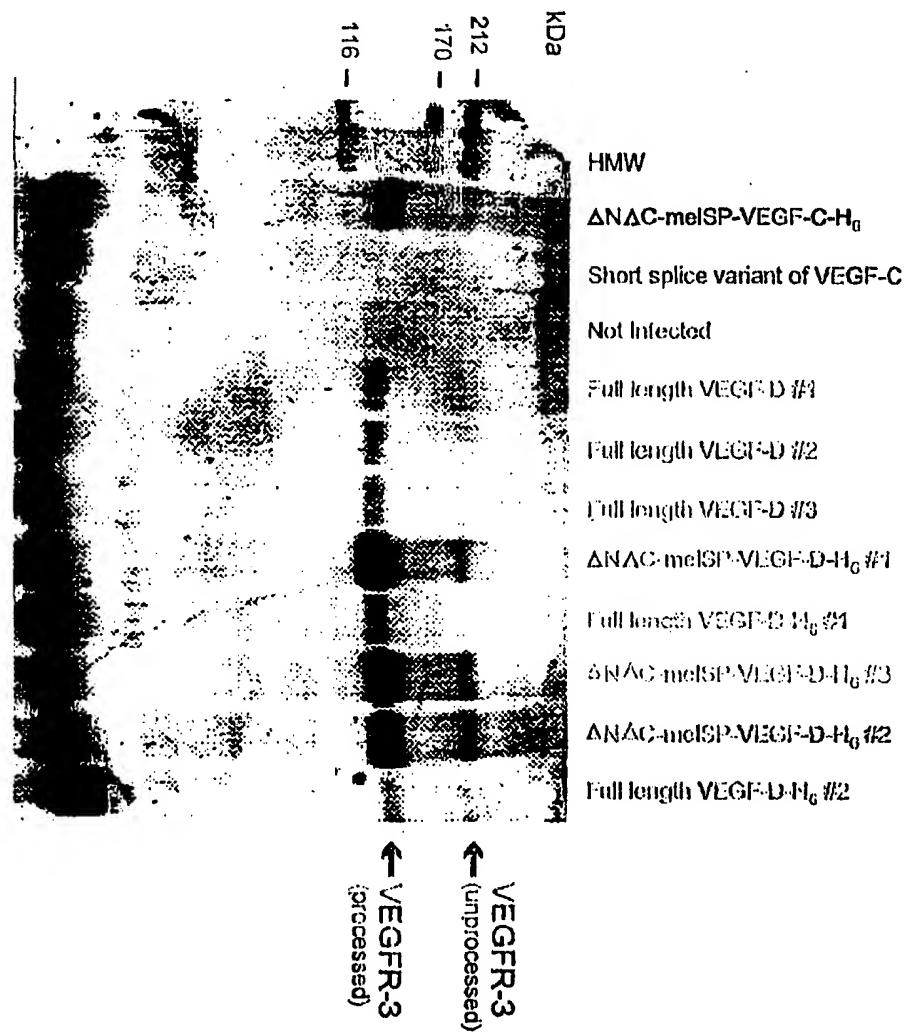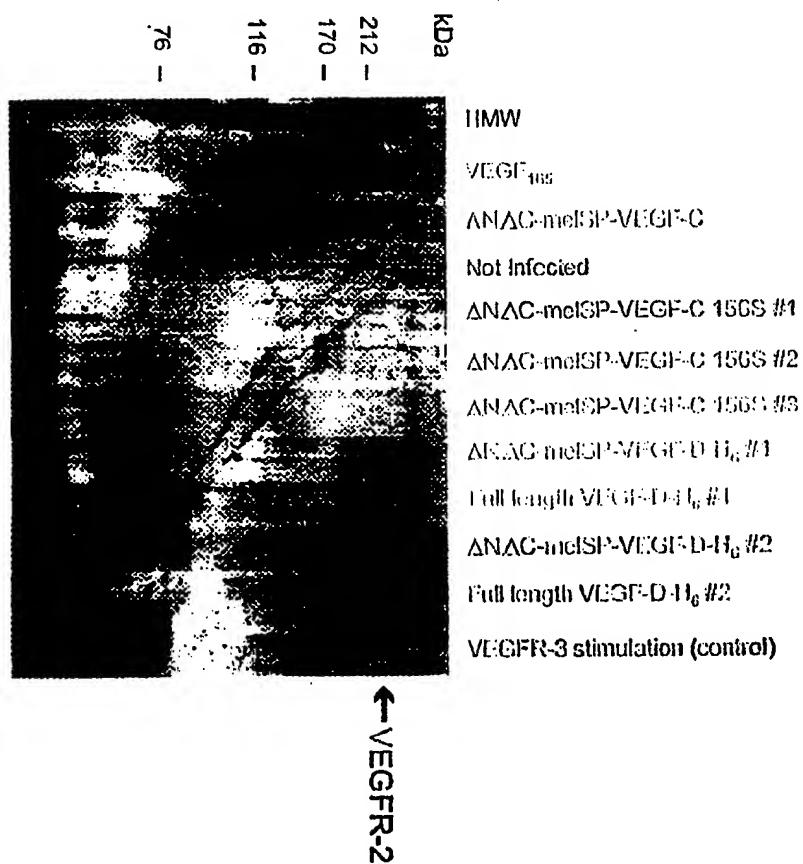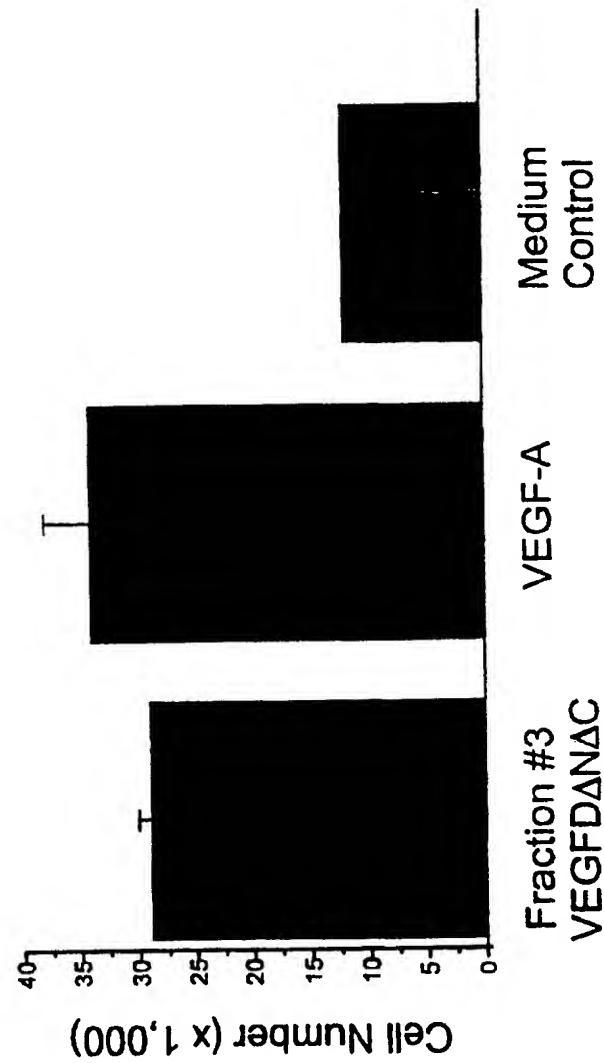
Expressed protein	VEGF-A	VEGF-D	VEGF-D*
I/P Antibody	anti-VEGF-A	M2	M2*

FIG. 12a.

FIG. 12b

FIG. 13

FIG. 14

FIG. 15

FIG. 16

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6)   :C12N 1/21, 5/10, 15/18, 15/63; C07K 1/14, 14/475, 14/49
US CL   :435/69.4, 325, 243, 320.1; 530/399, 412; 536/23.51
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/69.4, 325, 243, 320.1; 530/399, 412; 536/23.51

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database GenBank, Accession Number H24828, HILLIER et al. y142g02.r1 Homo sapiens cDNA clone 160946 5', 07 July 1995, see entire document.	6
X,P	ORLANDINI et al. Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. Proc. Natl. Acad. Sci. USA. October 1996, Vol. 93, pages 11675-11680, see especially Figure 1 and "Materials and Methods" at pages 11675-11677.	1-3, 5-6, 8, 16-23, 33, 35
A	TISCHER et al. Vascular endothelial growth factor: a new member of the platelet-derived growth factor gene family. Biochm. Biophys. Res. Comm. Decemer 1989, Vol. 165, No. 3, pages 1198-1206, see entire document.	1-13, 16-28, 33-35

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
. 29 OCTOBER 1997	2 3 DEC 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer CHRISTINE SAOUD
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*
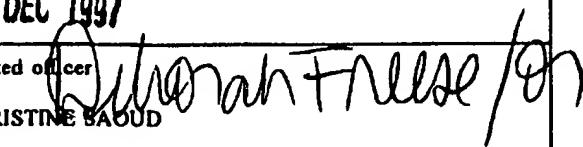
INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14696

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 14-15, 29-32, 51-57, 59-62, 68
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable
   claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers
   only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-13, 16-28, 33-35

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

   ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN, BIOSIS, MEDLINE, EMBASE, WPIDS
search terms: vascular endothelial cell growth factor, VEGF, PDGF, VEGF-D, endothelial cell proliferation, vascular permeability

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-13, 16-28, 33-35, drawn to DNA, vectors, host cells, polypeptides, pharmaceutical compositions of polypeptides and methods of making polypeptides.

Group II, claim(s) 36, drawn to a vector comprising antisense molecule.

Group III, claim(s) 37-45, 63-66 (in part), drawn to methods of promoting cell differentiation and/or proliferation.

Group IV, claim(s) 39-40, 63-66 (in part), drawn to methods of stimulating vascular permeability.

Group V, claim(s) 46-49, drawn to methods of inhibiting by administration of an antagonist which is an antibody.

Group VI, claim(s) 50, drawn to a method of inhibiting expression by administration of an antisense vector.

Group VII, claim(s) 58, drawn to methods of detecting VEGF-D.

Group VIII, claim(s) 67 (in part), drawn to a diagnostic test kit which uses an antibody.

Group IX, claim(s) 67 (in part), 69, drawn to a diagnostic test kit which uses primers.

Group X, claim(s) 70, drawn to methods of detecting aberrations in VEGF-D.

Group XI, claim(s) 71 (in part), 72, 73, drawn to a VEGF-D antagonist which is an antibody and pharmaceutical compositions thereof.

Group XII, claim(s) 71 (in part), 74, drawn to a VEGF-D antagonist which is a fragment or analog of VEGF-D.

Group XIII, claim(s) 76, drawn to methods of using a VEGF-D antagonist.

Group XIV, claim(s) 75, drawn to a method of increasing circulation and/or gas exchange.

Group XV, claim(s) 77, drawn to a method of treating intestinal malabsorption.

The inventions listed as Groups I-XV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical feature of Group I, DNA encoding a VEGF polypeptide, is not shared by all of inventive groups. Therefore, the main inventive groups is the DNA encoding a VEGF polypeptide, including the vectors and host cells containing said DNA, the polypeptide made with said DNA and the first method of using the DNA, which is the method of making the protein. The special technical feature of Group II is the vector containing the antisense. The special technical feature of Group III is the method of promoting cell differentiation and/or proliferation. The special technical feature of Group IV is the method of stimulating vascular permeability. The special technical feature of Group V is the method of inhibition by administration of an antibody. The special technical feature of Group VI is the method of inhibiting expression by administration of an antisense vector. The special technical feature of Group VII is the method of detecting VEGF-D. The special technical feature of Group VIII is the diagnostic test kit which utilizes an antibody. The special technical feature of Group IX is the diagnostic test kit which utilizes primers. The special technical feature of Group X is the method of detecting aberrations in VEGF-D. The special technical feature of Group XI is the VEGF-

D antibody. The special technical feature of Group XII is the VEGF-D antagonist which is an analog or fragment of VEGF-D. The special technical feature of Group XIII is the method of using an antagonist. The special technical feature of Group XIV is the method of increasing circulation and/or gas exchange. The special technical feature of Group XV is the method of treating intestinal malabsorption.

PCT Rule 13 does not provide for multiple products or methods within a single application. The methods are distinct because they have different goals, method steps, and/or starting materials as well as being qualitatively different methods. The products are distinct because they do not share the same or corresponding technical feature (i.e. DNA encoding VEGF-D) and because they are structurally different products.